

Spanish science is still at risk

Although it may well be the only country in Europe to increase research spending next year, Spain still faces the loss of outstanding researchers. Both the government and universities need to make difficult choices.

Spanish scientists like to quote the fact that the Brazilian footballer Ronaldo was this year sold by the club FC Barcelona to Inter Milan for a fee higher than the entire budget of their research council, CSIC. The national passion for football, it seems, is more enduring than that for science. Scientists are therefore right to be more concerned about the failure to capitalize on their country's recent investment in research than relieved to learn that a significant increase in research budget is foreseen for next year (see page 773). For the lack of research tradition makes Spain particularly vulnerable to the long-term effects of a period of famine.

In the 1980s, Spain, then one of Europe's lowest science spenders, injected a major funding boost. The research budget grew by around ten per cent a year, the number of research institutes expanded, and thousands of young scientists were sent abroad for postdoctoral training. The benefits of this push should now be being felt. But the economic doldrums of the 1990s threw the dream of matching the research efforts of countries such as Germany and France — rather than those of Greece and Portugal — off balance, and implicit promises that returning postdocs would be stably integrated into an expanding research scene were broken.

Corrective action must be taken before the present army of young scientists on which Spain's future depends leave for other careers. That haemorrhage is already under way, despite stopgap solutions offered by successive governments. A sustainable solution requires a

fundamental change to the outmoded and inflexible employment practices that Spain shares with some other European countries — particularly Italy. These continue to provide permanent researchers with all the privileges of tenure, even though these are no longer affordable, while outlawing the renewable temporary contracts that could allow the establishment of a tenure track system. The government has begun to debate changes in the law to increase employment flexibility in general, but changes are years away.

In the short term, the government must remember its promise to create 150 new tenured positions for CSIC out of the 1998 pool of new civil servant posts when these are distributed next spring, even at the price of taking jobs from other sectors; that would certainly raise morale among the ranks of mutinying foreign-trained postdocs. Research institutions must take advantage of the new rules of the National Plan which allow them to hire temporary staff on its grant money. And the academic community must help itself by accepting the need for greater mobility.

The latter means that universities must be prepared to abandon their tradition of hiring locally, and from within their own ranks, while young researchers must be prepared to leave their home town to compete for jobs, even temporary, that arise elsewhere. All this will inevitably involve some social dislocation; but it is part of the price that Spain must pay if it wishes to become a modern, science-based state. □

Light in dark places

Despite cultural differences, countries face common challenges in confronting new biomedical advances.

It is always a comfort when two individuals who have viewed each other sceptically, perhaps even disdainfully, from a distance find that, on closer acquaintance, they have more in common than they realized. Such was the experience in Paris last week at a meeting organized jointly by *Nature* and the British Council on the handling of bioethics issues in Britain and France. Those who were expecting a fiery clash between two opposing world-views will have come away disappointed. For beneath surface differences there was a surprising degree of harmony, particularly on the way in which the bioethics debate in both countries has opened up the question of public access to decision-making (see page 775).

Differences certainly remain. One focus of discussion, for example, was the status given to the concept of 'society' as an entity affected, for good or ill, by modern biomedical advances. France has, at least since the revolution of the eighteenth century, awarded greater significance to this idea in its legislation than Britain, which continues to think of communities primarily as collections of individuals. Some speakers suggested that this difference was responsible, for example, for contrasting attitudes about the extent to which threats such as eugenics can be adequately addressed by legislation.

But it also became clear that cross-Channel similarities are more important than differences. This in itself is not surprising. As has been graphically illustrated by the instant global reaction to the possibility of human cloning, the issues raised by modern science know

no national or political boundaries. At the same time, the growing displacement in all societies of traditional forms of personal contact by modern techniques of communication has added new weight to demands for transparency at all levels of decision-making.

For Britain, at least, the reluctance of government officials to make available the full details of technical reports relating to recent food scares, such as the outbreaks of bovine spongiform encephalopathy and *Escherichia coli* poisoning, has already strengthened the hands of those demanding that the Labour government fulfil its pre-election commitment to introducing a fully fledged Freedom of Information Act. In France, the needs are less obvious. But last week's meeting revealed a sense that more can still be done to engage the public directly in regulating the impacts of modern science — including giving the media greater access to this process.

Scientists, too, have a responsibility to open up. Three days after the meeting in Paris, it was announced through a British television company that researchers at the University of Bristol had produced a 'headless' frog embryo, opening the possibility of growing similar human bodies primarily as a source of 'spare parts'. To their credit, the scientists involved have not ducked from publicly discussing, even at this early stage, both the potential benefits and dangers of their work, including their own moral qualms. Their confidence that an informed public is a responsible public — more familiar as a political tradition in the United States — is welcome. □



Fusion researchers seek US role as partner in EU's JET facility

[WASHINGTON] A group of prominent US fusion scientists has suggested that the United States should try to become a partner in the European Union's Joint European Torus (JET) in the United Kingdom to gain greater access to the world's largest research facility for plasma physics.

The six physicists include the directors of the three main US centres for magnetic fusion research, at Princeton, San Diego and the Massachusetts Institute of Technology. They argue that formal US involvement in JET would improve their ability to use the UK facility to test and refine computer-based models for plasma confinement.

The idea has been put forward at a critical time, as the US government is in the process of deciding how to realign its fusion programme when a major international collaboration is completed next summer. The design phase of the International Thermonuclear Experimental Reactor (ITER), which absorbs about \$85 million of the US Department of Energy's \$230 million fusion budget, ends next July.

But the suggestion that the United States should offer to join JET at this stage has angered supporters of ITER on both sides of the Atlantic. They see it as a thinly disguised attempt to map out an alternative path for international collaboration in fusion research if ITER is not built.

The four ITER partners — Europe, the United States, Russia, and Japan — remain committed in principle to negotiating the construction of the machine next year, although the United States has already said that it will not play a large part in ITER construction, which is expected to be delayed (see *Nature* **387**, 746; 1997).

The physicists presented their proposal to join JET last month to a subpanel of the Fusion Energy Sciences Advisory Council (FESAC), the external body that advises the Department of Energy on how to run the programme. It is contained in a report on US strategy for exploring the science and technology of energy-producing plasmas.

The report recommends "support of ongoing ITER design and analysis, at a reduced level". But it also advocates "strongly increased US participation" at JET and at the Japanese facility, JT-60U. "We recommend that the US negotiates with Euratom for partnership status in JET," the proposal says.

Its authors, however, now concede that their call for negotiation was premature. "We meant to say 'explore,'" says David Baldwin, vice-president of fusion at General Atomics in San Diego, and the proposal's lead author.

Baldwin says that the proposal is not intended to divert resources from ITER, but to use JET to further refine the ITER design. He says that as well as supporting ITER, the US needs to undertake "dual-purpose" collaborative work that would advance fusion science in general, because Congress is unlikely to support further US contributions for ITER alone.

Hermann Grunder, director of the Thomas Jefferson National Accelerator Facility in Virginia and chair of the FESAC subpanel, says that FESAC strongly supported the strategic plan document. "JET is the one remaining facility that can give us scientific information on the burning of D-T plasma, in support of ITER," he says.

But his panel did not take up the recommendation to join JET. "It is a political hot potato, and we don't want to offend any of our European colleagues," says Grunder.

Charles Baker, the head of the US team working on the ITER design, says that extra US involvement at JET would be counterproductive if it also cut back on its involvement in ITER, as JET's research programme is devoted to supporting ITER.

At a full meeting of FESAC earlier this

week, the Grunder panel proposed allocating between \$10 million and \$20 million of the \$55 million now spent on ITER on physics collaboration at JET and JT-60U. The panel suggested that all but \$15 million of the US ITER money be spent on activities other than refining the current ITER design, including \$5 to \$10 million for "lower cost concepts" for achieving plasma burn.

Several FESAC members criticized the Grunder report, and the full panel, chaired by John Sheffield of Oak Ridge National Laboratory, was expected to back an interim plan more supportive of ITER than Grunder's. Francis Troyon, the chairman of the JET council, sent a message to Sheffield on Monday warning the United States that JET is busy with ITER-related experiments until 1999, and that thereafter "the future of JET is closely tied to that of ITER".

But Alan Gibson, deputy director of JET, says that informal discussions have already taken place about US membership of the facility, and adds that a formal proposal "of mutual benefit" would be welcomed. He says JET officials are keen to extend its operating life beyond 1999, but their plans are hampered by a shortage of funds. Colin MacIlwain

Cassini mission blasts off for Saturn



[WASHINGTON] Cassini, the most ambitious and expensive planetary expedition ever mounted, began its long trip to Saturn from Cape Canaveral, Florida, on 15 October (left). The spacecraft, which is costing the US, European and Italian space agencies \$3.3 billion to build, launch and operate, will arrive at Saturn in July 2004. It will orbit the planet for four years, investigating its atmosphere, moons, rings and electromagnetic fields.

In November 2004, the European-built Huygens probe will detach from the main ship and land on the large moon Titan, taking pictures and data as it parachutes through the thick atmosphere. Huygens is the European Space Agency's first planetary lander and the first designed to touch down on the satellite of another planet apart from Earth.

As a money-saving measure, Cassini will skimp on science during the first five years of its journey to Saturn, when it will swing past Venus, Earth and Jupiter to gain velocity. Unlike the Galileo Jupiter mission, Cassini's 12 science experiments will be turned off except for a gravitational wave detection experiment and occasional instrument health checks. Tony Reichhardt

AP/PETER COSGROVE

Nobel prizes honour atom-trappers...

[LONDON] For their development of methods to cool and trap atoms with laser light, this year's Nobel prize for physics has been awarded to Steven Chu of Stanford University, Claude Cohen-Tannoudji of the Collège de France and Ecole Normale Supérieure, Paris, and William Phillips of the National Institute of Standards and Technology (NIST) in Maryland.

"These three are absolutely the world leaders," says Peter Knight of Imperial College, London, adding that he is "hugely delighted" with the announcement.

Cooling and trapping atoms with light are distinct but related processes. Because optical traps for neutral atoms are generally shallow, the atoms must be cooled to below one kelvin before trapping can be contemplated.

Ions are easier because they interact more strongly with electromagnetic fields. "For a single ion in its much deeper [electromagnetic] trap, the attainment of ultra-low temperatures loses its importance," explains Hans Dehmelt of the University of Washington.

Transfer of momentum

Laser cooling of an atomic gas was proposed in 1975 by Theodor Hänsch and Arthur Schawlow of Stanford University. In the same year, David Wineland and Dehmelt suggested a similar scheme for cooling ions. Dehmelt's work on ions earned him and Wolfgang Paul of the University of Bonn a half-share in the 1989 Nobel prize; Norman Ramsey of Harvard University received the other half.

The principle behind the laser cooling of atoms is the transfer of momentum from a photon to an atom that can absorb it. The atom is given a kick in the direction in which the photon was travelling. Subsequent re-emission of a photon by the excited atom delivers a recoil impulse to conserve momentum. But if the emission is spontaneous rather than stimulated, the direction of the emitted photon is random.

A series of sequential absorption and emission events transfers momentum to the atom in the direction of the light beam, whereas the recoil force from emission averages to zero. The result is that an atom propagating counter to the light beam is slowing down, like a cyclist riding into the wind.

In the 1980s, Phillips and co-workers at NIST (then the National Bureau of Standards) used this idea to slow and cool beams of sodium atoms. The frequency of the cooling laser beam is tuned to just below that needed to induce an optical transition — the Doppler shift experienced by the counter-propagating atoms boosts the frequency to make absorption possible.

In 1985, Phillips and colleagues developed a method for trapping a cooled beam of sodium atoms magnetically, using a graduated magnetic field to shift the absorption frequency via the Zeeman effect and thus to compensate for the fact that the Doppler shift decreases as the atoms are slowed. They cooled the atom beam to less than 100 millikelvin, reducing the average velocity to almost zero.

But cooling a gas, rather than a beam, is harder, because atoms in a gas move in random directions. In 1985, Chu and co-workers, then at Bell Laboratories at Holmdel in New Jersey, used six laser beams arranged in mutually orthogonal pairs to cool a gas of about a million sodium atoms.

To cool all the atoms, each laser beam has to impart a force only to those atoms moving in the opposite direction. These atoms, however, are automatically selected because only they experience a Doppler shift sufficient to allow absorption.

The result is that the atoms experience a retarding force — a kind of viscosity — in whichever direction they move. Chu called this 'viscous' light field an 'optical molasses'.

Better ways of measuring the temperature of a gas held in optical molasses led Phillips to discover in 1988 that the gas could be cooled to 40 microkelvin — significantly lower than the predicted limit based on the Doppler effect. The finding was confirmed by other groups, including Cohen-Tannoudji's in Paris.

Cohen-Tannoudji made pivotal contributions to understanding how sub-Doppler cooling was possible. The key point is that there is a finite mean time between absorption events for each atom, during which atoms find themselves generally moving up energetic slopes in the electromagnetic field — a phenomenon called the Sisyphus effect.

Further limits to cooling

Although the Doppler limit can be overcome, it appeared at first that there was another fundamental limit to the maximum degree of cooling: atoms could not be slowed below the velocities attained by recoil when they emitted a photon. This is because even the slowest atoms are continually absorbing and emitting.

But in 1988, Cohen-Tannoudji's group demonstrated that even this limit could be surpassed. "You must prevent very cold atoms from absorbing light," he says, and to do that he uses quantum interference effects to convert these atoms to a dark, non-absorbing state.

Their initial experiments used two opposed laser beams to achieve sub-recoil cooling in one dimension; they extended this

to two dimensions in 1994 and to three dimensions, using six laser beams, in 1995. In this way they cooled a gas of helium atoms, for which the recoil limit is 4 microkelvin, to less than a fifth of a microkelvin.

In the practical sphere, the possibilities of laser cooling and trapping will be felt in precision measurement. More accurate atomic clocks that exploit laser cooling are already in operation, and Phillips foresees improved gyroscopic rotation sensors for aircraft navigation.

Chu has pioneered the use of laser beams to trap neutral particles other than atoms. Arthur Ashkin at AT&T Bell Laboratories first demonstrated this kind of optical trapping in 1970, using two counter-propagating beams to trap microscopic latex particles in water. It was later realized that a single beam would suffice, and Chu, Ashkin and collaborators achieved single-beam trapping of polymer particles in 1986.

That work makes this year's physics prize rare in having made a significant impact as far afield as the life sciences, where laser trapping and manipulation is used to study the mechanics of cellular machinery.

The techniques for cooling and trapping neutral atoms were essential for the long-awaited demonstration in 1995 of Bose-Einstein condensation (BEC) of atoms by Carl Wieman and co-workers at the University of Colorado, an experiment that had been widely tipped to net this year's prize.

But "one couldn't reward that without first recognizing laser cooling," says Knight, who admits to losing a bet on the award being made for BEC.

Philip Ball



Winners of the Nobel prize for physics: Chu (above), Phillips (top right) and Cohen-Tannoudji (right).

... and biologists' work on protein energy converters

[LONDON] The 1997 Nobel prize for chemistry has been awarded to three biologists for their work on proteins that interconvert chemical energy, in the form of the 'high-energy' compound adenosine triphosphate (ATP), and electrical energy — the asymmetric distribution of ions across biological membranes.

Jens Skou of Aarhus University, Denmark, receives half the prize for his discovery of the Na^+/K^+ pump. Paul Boyer of the University of California, Los Angeles, and John Walker of the UK Medical Research Council's Laboratory of Molecular Biology in Cambridge share the other half for their elucidation of the mechanism of ATP synthase. Between them, these three straddle more than 40 years of research in bioenergetics.

Although few doubt the researchers fully deserve such recognition, many were surprised by their presence on the same citation and by the timing of the award to Boyer and Walker. Many questions about the mechanism of ATP synthase — in particular its complete structure — remain unanswered.

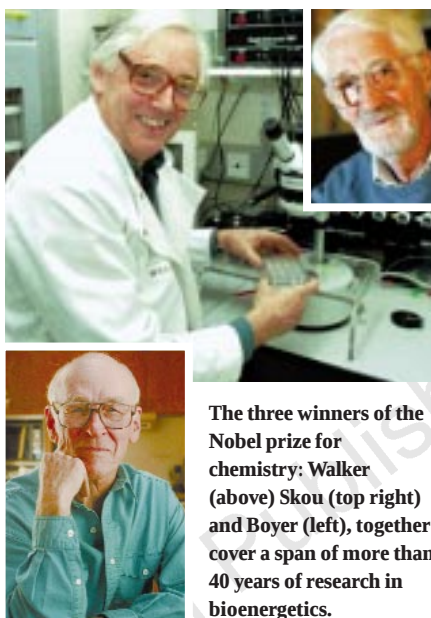
The distribution of the prize has also raised some eyebrows. Work on the sodium pump was "long overdue for recognition", says J. Clive Ellory of the University of Oxford's physiology department. But it had been expected that Skou would eventually share the prize with other prominent researchers in the field, such as Ian Glynn of the University of Cambridge and Robin Post of Vanderbilt University, Tennessee, who helped characterize the workings of the Na^+/K^+ pump.

Enzymes and energy

Skou's pioneering work in the late 1950s showed that an enzyme could take the energy supplied by ATP and use it to transport material across a cell's membrane. It had just been established that different concentrations of ions were maintained inside and outside cells, and Skou set about identifying the cause of this disparity by analysing the nerves of crabs, extracting cell membranes in which he identified an ATP-degrading enzyme stimulated by sodium ions.

Further investigation showed that this protein comprised two subunits and, at the cost of one molecule of ATP, moved three sodium ions from the inside of the cell to the outside and two potassium ions from the outside to the inside.

Boyer and Walker attacked a reciprocal problem. Rather than study how ATP creates ion gradients across cell membranes, they looked at how the energy in these gradients can be used to synthesize ATP from its low-



The three winners of the Nobel prize for chemistry: Walker (above) Skou (top right) and Boyer (left), together cover a span of more than 40 years of research in bioenergetics.

energy form, adenosine diphosphate (ADP).

Peter Mitchell, working at the Glynn Laboratory in Bodmin, Cornwall, had shown in the 1960s that the ultimate result of metabolism — the breakdown of an organism's food source — was to produce a difference in hydrogen ion concentration across the cell's mitochondrial membrane, work for which he received the Nobel prize in 1978.

Boyer worked through the 1970s and 1980s investigating ATP synthase, the enzyme that uses this gradient to synthesize ATP. It was soon discovered that this enzyme consisted of a large number of proteins, but that it could be separated into two parts: F_0 , which was tightly bound in mitochondrial membrane, and F_1 , which contained the ATP binding and synthesizing activity.

Concentrating on F_1 , Boyer showed that it was made of three copies, each of two similar subunits, α and β , of which β bound the ATP. But the subunits were never in the same state — one bound ATP and one ADP, while the third had an empty binding site.

Boyer proposed that the subunits changed between these conformations in a concerted fashion, constantly cycling from empty to ADP-bound to ATP-bound in what he called the "binding change mechanism".

There was one other component of F_1 , the γ -subunit, of which there was only one copy. Boyer proposed that this γ -subunit rotated within the other subunits like a crankshaft in a three-cylinder motor. "Why Paul's achievement is so praiseworthy," according to Richard Cross of the State University of New York Health Science Center, is that these "parts of the mechanism flouted dogma".

Indeed, it has taken almost all of the 16 years since the mechanism was fully laid out to establish its veracity. The greater part of this proof has come from Walker, who started out in the 1980s to provide a clear picture of the enzyme and its action.

He began by determining the sequences of the various subunits using low-resolution electron microscopy, which showed a 'lollipop'-shaped protein sticking out from the mitochondrial membrane. By 1994, he had elucidated the atomic detail of F_1 using X-ray crystallography (see *Nature* **370**, 621–628; 1994). This structure more than bore out the predictions of Boyer, showing an elongated γ -subunit pushing against one of the α/β pairs, forcing the three to adopt their differing conformations.

Still unknown are the structure and action of F_0 , which somehow uses a flow of hydrogen ions across the mitochondrial membrane to drive the rotation of F_1 . Work is well under way to determine this structure, and it would be unwise to bet against Walker solving this as well.

Without such information, has the Nobel committee been premature in its award? David Hackney of Carnegie Mellon University, Pittsburgh, is certain that it has not. "It is recognition of something that Paul [Boyer] has worked his entire life on," he says. "All his propositions, which were controversial at the time, have turned out to be true."

Demonstration of rotation

The most controversial of these propositions was that F_1 rotates and it may be that a graphic demonstration of this earlier in the year triggered the presentation of the prize.

Richard Cross and Wolfgang Junge of Osnabrück University had separately provided indirect evidence for the rotation, but it was Masasuke Yoshida of the Tokyo Institute of Technology who, by attaching a filament to the γ -subunit, was able to film the rotation in action (see *Nature* **386**, 299–302; 1997). Cross is convinced this was a great influence on the Nobel committee. "When he [Yoshida] showed that film at meetings, people's jaws dropped," he said.

Hackney recalled it as the "only time that a platform session [was] stopped due to applause from the floor". He has no doubt about its impact. "The biochemical data were sufficient to convince people in the field [about Boyer's mechanism] but the visualization of the spin was the last nail to finalize it."

What are the prospects of the same level of structural information emerging to illuminate Skou's Na^+/K^+ pump? Gene Scarborough of the University of North Carolina is working on the problem and hopes a structure will emerge within five years. He has no doubts about the rightness of the award to Skou. "When I teach this stuff [bioenergetics] I always begin with Skou, because that's where it begins." Christopher Surridge

Faulty solar panel jeopardizes Mars mapping project

[WASHINGTON] A faulty solar panel on the US Mars Global Surveyor spacecraft has led mission managers to suspend normal operations, and may jeopardize their goal of mapping the entire planet at high resolution.

Project managers raised the craft's orbit to a safe altitude on 12 October after one of two electricity-producing panels moved unexpectedly while experiencing drag in the martian atmosphere.

Since arriving at Mars last month, the spacecraft has been repeatedly dipping into the atmosphere to lower and circularize its elliptical orbit, a technique known as 'aerobraking'. But on 6 October the atmospheric density suddenly doubled. The solar panel, which engineers had thought was stuck open at 20 degrees from its intended latched position, moved first to the latched position, then past it.

Engineers at the Jet Propulsion Laboratory in Pasadena, California, and Lockheed Martin Astronautics in Denver, Colorado, which built the spacecraft, will spend two weeks or more trying to duplicate the solar array's behaviour using a replica on the ground to try to understand the problem.

Mars Global Surveyor is meant to end up in a nearly circular orbit averaging 378 km above the surface of Mars. Mapping had been due to begin in March and to last for 687 days, or one full martian year. Even if no more aerobraking is allowed, it may be possible to use the spacecraft's fuel to lower its altitude, but it is unlikely that the optimum mapping orbit could then be achieved. Science instruments continue to collect data while engineers are studying the solar panel problem.

If the craft remains in an elliptical orbit, it will be too far from the surface of Mars for high-resolution imagery, so observations of the planet's southern hemisphere will suffer, according to Michael Malin of Malin Space Science Systems in San Diego, California, which built the spacecraft's camera system.

Some damage has already been done. The plan was for the craft to pass over the martian equator at the same time every day, two o'clock in the local afternoon. Due to the hiatus in aerobraking, that orbit is no longer possible, says Malin, so other options are being considered.

If crossing the equator shifts too much towards midday, the shadows become shorter, and the definition of surface features would be washed out in the photographs. Malin says a mid-morning equator crossing is preferable for imagery, but is not ideal for thermal measurements of the surface, which are better done in the afternoon after the planet has warmed up.

Tony Reichhardt

Genome research strategy splits Japanese scientists

[TOKYO] Japanese government officials are said to be trying to resolve a dispute about strategy between researchers taking part in the Genome Frontier Programme, a project backed by the Science and Technology Agency (STA) and the Institute of Physical and Chemical Research (RIKEN).

The ¥4-billion (US\$33-million) project, due to start in October next year, had been intended to be based on new techniques that make use of full-length complementary DNAs (cDNAs) for DNA base sequencing, elucidating gene and protein function, and analysing protein structure. The use of full-length cDNA allows key parts of the clone to be targeted for sequencing, as well as direct production of proteins from the cloned cDNA.

But some leaders of Japan's human genome research who have been advising on the programme are said to oppose this strategy, and to be insisting that the programme be restricted to DNA sequencing.

"The formal description of the project is that it will involve both DNA sequencing and protein-related research, but this is very unlikely to happen," says a leading genome researcher at Kyoto University. "The protein-related research will probably have to be carried out under a different project."

The research strategy proposed by RIKEN was planned to combine three projects: sequencing a full-length cDNA using the latest automated high-speed DNA sequencer; producing a genetic 'encyclopaedia' of gene and protein functions; and analysing protein structure by using nuclear magnetic resonance (NMR) at the 'NMR Park' that the STA wanted to set up (see *Nature* **381**, 815; 1996).

But many scientists now fear the RIKEN programme may become little more than an extension of a DNA sequencing project carried out at Japan Science and Technology Corporation, formerly the Japan Information Centre of Science and Technology.

"A national project like this should consider its accountability to the taxpayers," says

Akiyoshi Wada, former dean of science at Tokyo University and the director of Sagami Chemical Centre, a privately funded institute which has filed Japan's first applications for patents on human genes (see *Nature* **361**, 815; 1996). "DNA sequencing alone will not contribute to the understanding of gene function; only through protein-related research can we obtain patentable products for medical use and research."

A member of the Japanese Diet (parliament) is said to have intervened in the STA budget plan, introducing a requirement that the agency should carry out only DNA sequencing research, which he considers an "international trend", and postponing protein-related research as being "premature".

But one project leader at RIKEN claims that, if the proposed projects are abandoned, Japan will miss out on a chance to join the front rank of international genomics research. "DNA sequencing is already being carried out in Europe and the United States, and we are relatively behind in this field." People in Japan, he says, are reluctant to enter new areas in genetic research, which is still dominated by the 'traditionalists'.

But another prominent leader of Japanese genome research supports placing an emphasis on DNA sequencing, on the grounds that the Japanese effort in human genome research is not sufficiently advanced. "Although supporters of the protein-related research emphasize the need to compete internationally, that is only to attract funding from the Ministry of Finance," he says. "Realistically, such an unprecedented project should be considered as a potential project for the future."

Wada, who argued strongly for automated and high-speed DNA sequencing ten years ago, is bitter about where the Genome Frontier Programme may be heading. "My suggestion was ignored in Japan ten years ago, and this is the main reason why we are lagging behind other countries," he says. "We cannot be left behind again." Asako Saegusa

Launch of Lunar Prospector delayed

[WASHINGTON] The launch of a low-budget US spacecraft mission to map the chemical composition and gravitational field of the Moon and search for evidence of ice has been delayed for the second time.

The \$63-million Lunar Prospector will now leave Earth no earlier than 5 January, due to delays in developing its new Athena 2 launch vehicle.

The launcher and spacecraft are built by

Lockheed Martin Corporation. Lunar Prospector's launch was originally scheduled for September, then November. If the date slips past February, it could compromise the mission's ability to meet all its scientific objectives, according to the principal investigator, Alan Binder of the Lunar Research Institute in Gilroy, California. The current plan is to complete the main mission before a lunar eclipse in July 1999.

T. R.

Spanish budget raises postdoc job hopes

[BARCELONA & MADRID] After a number of lean years, Spanish science is set to expand. The government's proposed budget, which will be presented to parliament this week, contains significant funding increases to both basic and applied research.

Funding for Spain's National Plan, which provides money for targeted research, will be increased by eight per cent, and the national research council CSIC, which runs 96 basic research institutes, by nearly six per cent. At the same time, by dangling the prospect of new research positions, the government is offering a glimmer of hope for Spain's army of foreign-trained postdoctoral fellows, hundreds of whom face unemployment by the end of the year.

The proposed increases go some way to re-establishing Spain's efforts in the 1980s to create a strong research base. Research spending was doubled by 1990, even though the level reached — 0.84 per cent of gross national product — was still well short of the European Union's mean of 1.9 per cent, Spain's ultimate goal.

Research budgets have since stagnated, and next year's planned rise would in real terms return the budget only to its 1994 level. The election promises of the right-wing government, which ended the 13-year run of socialist rule in May last year, that it would restart growth in research spending, were not kept for 1997. But the upturn in Spain's economy has eased the brakes.

The change in fortunes will mean more temporary jobs for scientists through the National Plan, which for the first time will allow its grants to be partly used for payment of salaries. The budget will not in itself mean more permanent research positions. But the government has also promised to support a request from CSIC's president, César



Aldana: stresses industrial links.

Nombela, for the creation of 150 new CSIC research positions next year. Salaries for permanent researchers come from a central pool reserved for all civil servants. This year, CSIC managed to secure 10 per cent of all new positions earmarked for highly qualified personnel. But because of the strict limit on total numbers, these amounted to only 30 junior, and 20 senior, posts.

Jesus Maria Gonzales, the new secretary of state for universities and research, says that CSIC is likely to do better in 1998. "The government is prioritizing new research positions, and I very much favour plans to introduce new blood into CSIC," he says.

The new positions, if they materialize in full, will help alleviate the problem of the hundreds of young scientists sent abroad on research fellowships in the 1980s as part of the plan to build up national research expertise. They returned as skilled scientists, only to find that the bubble had burst. There were few openings in CSIC, while universities had developed staffing problems and were not hiring staff from outside their own institutions (see box below). Industry showed little interest in hiring specialized scientists.

In 1992 the government made a one-off offer of 1,400 three-year contracts, hoping that this would solve the problem. When it did not, 100 of these were extended for up to two years. At present, 20 per cent of CSIC scientific staff are on such contracts, all of which are now coming to an end.

The contracts are linked to research grants held by tenured members of public research institutes, a point that has been

criticized by young scientists and many of their senior colleagues, who have been campaigning for independent research positions. Nombela would like to change the career structure of CSIC to make such career track positions possible. "But it can't work like that in Spain at the moment," he says. The government is reviewing the inflexible system of civil service employment, but reform is unlikely in the next few years.

Disillusioned young scientists are starting to leave research. Monserrat Vendrell, for example, who worked as a postdoctoral fellow for four years at the Roche Institute in the United States before returning to Spain in 1992, has recently left research for a more certain career in science communication. Josep Casalviserta, who has been surviving on contracts at CSIC institutes in Barcelona since returning from France in 1995, says he will leave research next year if the proposed new openings at CSIC do not materialize.

Many established scientists are upset at the threat the loss of expensively trained personnel could pose to the future of their country's science. According to Mariano Estaban, director of CSIC's National Centre for Biotechnology in Madrid, the push for science in the 1980s went far towards establishing the international credibility of Spanish researchers. Credibility will be lost for ever, he fears, if that investment is now dissipated.

But several temporary measures are being set up. In addition to enabling the National Plan to give grants covering personnel costs, the government has made the plan responsible for administering a Pts28 billion (US\$190 million) grant from the European Social Fund — part of the European Union's structural funds for subsidizing poorer regions — which is intended to forge links between academics and industry.

Part of this money, which must be spent in 1998 and 1999, will subsidize the salaries of up to 200 young scientists, each within six years of completion of his or her PhD, to work in research in industry. A similar scheme launched last year by the government of the autonomous region of Catalonia resulted in two-thirds of participating young scientists being offered permanent positions in their companies.

Fernando Aldana, director of the National Plan, has little sympathy for the plight of the postdocs trained abroad. "They make a lot of noise, but their numbers are small," he says. Reflecting Spain's new emphasis on research with immediate industrial applicability, and the tensions this has created between basic researchers and policy-makers, he says that Spain's priorities should not be dictated by the skills the postdocs acquire abroad.

Alison Abbott

University rectors join task force on reforms

[BARCELONA & MADRID] Spanish university rectors last week accepted an offer from Esperanza Aguirre, the education and culture minister, to take part in a task force with ministry officials to clarify the main problems facing Spanish universities, namely the control of university curricula and the employment structure of academic staff.

The agreement could end a long battle within the ministry, and between the minister and the Spanish University Rectors'

Conference. Last July saw the resignation of Fernando Tejerina, secretary of state for universities and research, who had sided with the rectors, and three other senior ministry officials.

Universities are frustrated by Aguirre's unwillingness to discuss reforms to laws on universities passed in 1983. The rectors want a modified law to enable them to take over control of curriculum design from central government, and to regularize the employment conditions of 23,000 teaching

staff hired on short-term contracts in the 1980s. The rectors also want a system of tenure track positions.

Aguirre has promised to issue a universities' policy by the end of the year. According to the new secretary of state for universities and research, Jesus Maria Gonzales, this is likely to include changing academic selection procedures to encourage more competition between universities, and to recruit outside their own institutions and localities.

A. A.

Genome study maps chemical sensitivity

[WASHINGTON] Details of a \$60-million study of how 200 genes affecting susceptibility to chemicals vary across the population of the United States were unveiled last week at a conference organized by the US National Institute of Environmental Health Sciences (NIEHS).

During the meeting, held at the National Institutes of Health in Bethesda, Maryland, experts from fields including population science, genetics and epidemiology discussed the strategy of the Environmental Genome Project (EGP), which will study the DNA of about 1,000 individuals.

The meeting also heard concerns that genetic variations discovered by the project, and later found to be associated with disease risks, could be used to discriminate against individuals and groups, barring them from jobs and life or health insurance.

Commonly occurring variations (polymorphisms) in the genes that encode proteins which detoxify chemicals, repair DNA or regulate cell death are thought to increase the susceptibility of their carriers to damage from environmental toxins or carcinogens.

The EGP will seek to document the occurrence of selected polymorphisms. In a second stage, scientists will try to discover exactly how each polymorphism, combined with environmental exposures, increases disease risk. Ultimately, the goal is prevention, by allowing, for example, public health

measures to be targeted more accurately at susceptible subpopulations.

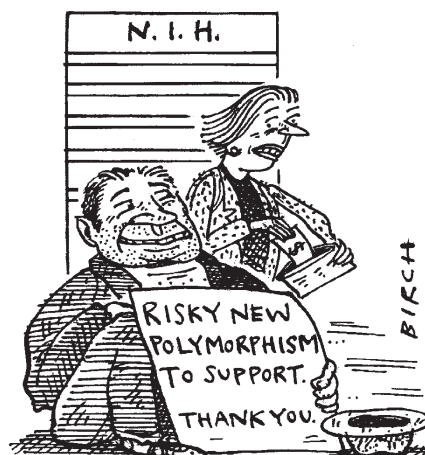
"More precise information would permit the best protection at the least cost," says Kenneth Olden, the director of NIEHS, who predicts that the project will have "a tremendous public-health impact".

The EGP does not aim to link polymorphisms to specific disease proclivities; that, NIEHS officials say, is a future project for extramural scientists, using the project's findings. Instead, it will seek to identify and catalogue the nature and distribution of the polymorphisms, and make the information available on an Internet database.

But the task is beset by complex and sometimes contentious questions, for example how to choose the most appropriate 200 genes to study (NIEHS has begun by soliciting gene nominations in journal advertisements), how to select the racial make-up of the sample population, and how to decide whether information on ethnic origins should be identifiable on the database.

NIEHS says that the database should not reveal this information. According to one conference participant, however, many scientists "think that's a really bad idea" because it would waste valuable data that could be used to help subpopulations.

But Francis Collins, the director of the National Human Genome Research Institute (NHGRI), who spoke at the conference on



the project's ethical implications, disagrees. He says that while the goal may be laudable, ethically it is a minefield.

"How would Native Americans feel about having their DNA samples in this reference set and having somebody decide they want to look at genes that might be involved in alcoholism?" said Collins. "Would that be well received?" The NHGRI will host a conference in early December on how an appropriate reference set could be designed.

Charles Langley, a population geneticist at the University of California, Davis, says a separate concern is providing much of the impetus behind the EGP — and other, increasing efforts to identify polymorphisms throughout the genome. This is the fear that industry will begin to patent polymorphisms that could be useful markers for disease genes and drug sensitivities.

Those scientists opposed to such patenting say this would ultimately inhibit medical research and development. They hope that the EGP will pre-empt industry by rapidly identifying key polymorphisms of environmental relevance and making them public. "All of these variations have to be ultimately public. They can't be licensed, they can't be patented," insists David Cox, a geneticist at Stanford University who addressed the conference.

Cox says he has been personally told that the pharmaceutical company Glaxo has no interest in such patents. At a meeting last month of the NHGRI's Advisory Council, Alan Williamson, vice president for worldwide research strategy at Merck & Co., implied the same.

Olden has proposed spending \$60 million on the EGP over six years, beginning in 1998. In a second phase, NIEHS will enlist extramural researchers nationwide to discover exactly how the polymorphisms interact with environmental exposures to cause — or sometimes forestall — disease. That understanding, it is hoped, will lead to preventive measures. Meredith Wadman

Diversity project 'does not merit federal funding'

[WASHINGTON] The proposed international Human Genome Diversity Project (HGDP) is not yet sufficiently feasible or well-defined to merit support from US government agencies, according to a study by the National Academy of Sciences.

The study, by a panel chaired by Jack Schull of the University of Texas Health Center at Houston, says the diversity of the human genome is of importance from both the anthropological and the biomedical point of view. But it says that the main focus of the study — a 'consensus document' prepared in 1993 by a human genome diversity subpanel of the Human Genome Organisation — meant different things to scientists from the two disciplines.

"Different participants in

the formulation of the consensus document had quite different perceptions of the intent of the project, and even of its organisational structure," the panel found.

It therefore suggests that the National Institutes of Health and the National Science Foundation — the two agencies that asked for the report — should confine support of human genome diversity work to projects inside the United States. The panel says they should hold discussions with foreign agencies about how international projects should be structured before supporting any international work.

The academy's report is another setback for advocates of HGDP such as Luigi Luca Cavalli-Sforza, a population geneticist at Stanford University, who

conceived the idea. Two years ago, the project received a cool response from the United Nations Educational Scientific and Cultural Organisation (see *Nature* 377, 373; 1995).

Critics say that the project would exploit genetic information obtained from people of confined gene pools — for example, from small, isolated tribes — without giving anything back. They maintain that the information obtained from such studies could lead to genetic discrimination.

The academy found that the work the project would do has "substantial scientific merit and warrants support", but that ethical, legal and human-rights concerns, as well as organizational ones, must be met before it can proceed. Colin Macilwain

'Bioethics needs better input from public'

[PARIS] The biggest challenge facing those who have to grapple with bioethical issues is to find more sophisticated ways of involving the public in decision-making. There is a need to improve the supply of information, to make decision-making procedures more open and to make the bodies responsible for decisions more representative of society.

This was one message to emerge from a one-day conference, 'Too hot to handle?', organized in Paris last week by the British Council and *Nature*. The meeting, attended by leading scientists, legal experts and politicians, explored the similarities and differences between the French and British approaches to resolving bioethical issues, and considered how to reach international agreement on such matters.

Different historical and cultural traditions have resulted in France tending to legislate on matters that would usually be regulated in the United Kingdom through codes of conduct (see *Nature* **389**, 661; 1997).

Another stark difference is that French thinking generally starts out by trying to balance the rights of the individual with those of society. Jean-François Mattei of the Hôpital d'Enfants de la Timone in Marseilles, and a member of the French National Assembly, pointed out, for example that a ban on prenatal diagnosis of embryos was supported during parliamentary debate of France's 1994 bioethics legislation on the basis that, while it might represent progress for individuals, this was outweighed by the risk that it might become "systematic and collective", and encourage eugenics.

Mattei, a leading architect of France's bioethics legislation, said that society had a responsibility to ensure that the brutal eugenics of the past is not replaced with "gentle medical eugenics" and the "elimination of those not meeting a genetic norm".

But British speakers remarked that little is heard of the 'individual-versus-society'

debate in the United Kingdom, where the rights of the individual and family are pre-eminent. David Porteous, from the Medical Research Council's Human Genetics Unit in Edinburgh, spoke for many when he argued that Britain considers that abuses can be adequately prevented by the "well-worked" democratic apparatus.

Almost all delegates, however, agreed that the days of debate on scientific advances affecting society being largely restricted to expert committees are numbered. "It is not science which will determine how genetics is applied," asserted Porteous. He said that policy-making now demands a more sophisticated approach.

Derek Burke, chairman of the UK Advisory Committee on Novel Foods, agreed that it is "no longer sufficient for governments to take the advice of an expert committee and to expect the public to accept the conclusions without question".

A succession of speakers reiterated that public confidence in policy will emerge only if the decision-making process is made more open. Pressure has grown for expert advisory committees to include consumer representatives, to make the minutes of their meetings public and to include minority viewpoints in their conclusions.

Trust is the key, said Tony Burton, deputy director of the UK Consumers' Association, arguing that "consumers are perfectly capable of making informed choices about risk, provided all the conditions are there. The need is to create the conditions." The European public is not against genetically modified organisms, he argued. "A minority are, but the majority are just confused."

Burke, whose own committee has included consumer representatives for six years, agreed that consumers provide valuable input by bringing different "value systems" and perspectives to those of experts.

The creation of national bioethics com-



Mattei: French emphasis on social rights.

mittees represents a "revolutionary" leap in the process of democratizing decision-making, argued Nöelle Lenoir, a member of France's constitutional court and chair of the bioethics committees of the European Commission and the United Nations Educational, Scientific and Cultural Organization. She said that these differ radically from expert advisory committees and statutory regulatory bodies, such as the UK Human Fertilization and Embryo Authority, in that their members are chosen with the aim of creating a multicultural and multidisciplinary forum.

On such panels, scientists are confronted with philosophers, legal experts, physicians and theologians, reflecting the major faiths and main streams of thought in society.

But most delegates, including Lenoir, agreed that bioethics committees should not have a monopoly on ethical issues, and should promote public debate. Several speakers warned that bioethics committees should refrain from offering "solutions" to ethical problems but rather focus on clarifying the issues. "The deliberations of ethics committees are as important [as their conclusions]," said Lenoir.

But some speakers expressed reservations that bioethics committees are excessively elitist and not sufficiently representative of public opinion. There was concern that pressure on such committees to reach conclusions could force an unnatural consensus that masked dissenting opinions.

British speakers also displayed scepticism about such committees, preferring the UK system where a wide diversity of bodies make recommendations on ethical issues. Burke said he preferred the British "muddle" to the "pontification" of ethics committees, arguing that "dissent is essential".

Lenoir, however, reckoned that bioethics committees are becoming the model of choice; eight member states of the European Union have created such committees, and two more have announced plans to do so. Jean-Yves Nau, medical correspondent of *Le Monde*, called for the French national bioethics committee meetings to be opened to the press and the public.

Declan Butler
An edited version of the proceedings will appear on Nature's web site.

Nobel backing for alternative health journal

[WASHINGTON] Fifty Nobel prizewinners are lending their prestige to a new journal aimed at critically evaluating the claims of alternative medicine. *The Scientific Review of Alternative Medicine*, described as "the first peer-reviewed journal dedicated entirely to the scientific, rational evaluations of unconventional health claims", was launched in Washington DC last week. The Nobel laureates, including Baruj Benacerraf, Francis Crick, Arthur Kornberg, Leon Lederman and Glenn T. Seaborg, calling themselves the Council for Scientific Medicine, say that "the need for objective, scientific critiques of the claims of

'alternative' medicine has never been greater".

The biannual journal is published by Paul Kurtz, founder of Prometheus Books. Kurtz, formerly a professor of philosophy at the State University of New York at Buffalo, also publishes *Skeptical Inquirer*, the journal of the Committee for the Scientific Investigation of Claims of the Paranormal, based in Amherst, New York. The first issue of the new journal deals with practices such as chelation therapy ("ineffective"), therapeutic touch ("pseudomedical"), and homeopathy ("by every objective, rational, and medical standard, homeopathy has failed to establish its scientific credibility"). Meredith Wadman

Argentina backs US on developing country greenhouse targets

[LONDON] Argentina has endorsed a US proposal that the faster-growing developing countries should join industrialized countries and make legally binding commitments to reducing greenhouse gas emissions. The endorsement comes on the eve of negotiations in Bonn, Germany, this week to discuss the text of a greenhouse gas protocol that will be finalized at the annual conference of the United Nations climate convention in Kyoto, Japan, in December.

Argentina is the first of the Group of 77 developing countries to openly break ranks; most continue to oppose the US proposals, and deadlock threatens to derail the Kyoto talks because the protocol needs a consensus of all the climate convention's signatory countries.

Some European Union member states are warming to a compromise solution in which developing countries should begin to discuss emissions reductions during the Kyoto talks, perhaps even pencilling in a future date for legally binding targets.

Meanwhile, the issue of developing country commitments will be discussed at the Commonwealth heads of government meeting in Edinburgh, UK, which starts tomorrow (24 October), and during a week-long visit to the United States by China's president Jiang Zemin. China is known to favour an emissions reduction protocol formulated on a per capita basis.

Top German universities win most funding

[MUNICH] Ninety per cent of grant money given by the Deutsche Forschungsgemeinschaft, which funds university research in Germany, was awarded to just 50 per cent of universities in the first half of the 1990s. Information about the relative performances of German universities in winning grants was published for the first time last week, as a way of promoting competition between them.

Clinton shoots down asteroid mission

[WASHINGTON] US President Bill Clinton last week used his new 'line-item' veto powers to delete funding for 13 military projects, including the controversial Clementine 2 spacecraft mission that was to have flown past two or more asteroids from 2000. The project now appears dead, two years after it was first included in the Pentagon budget.

Clementine 2 was to have tested miniature spacecraft components left over from the 'Star Wars' Strategic Defense Initiative. It would have sent microsatellites crashing into

the asteroids' surface to study the crater and ejected material in an attempt to learn more about asteroid composition.

There was enthusiasm for the mission in some parts of the US Air Force and cautious interest from the National Aeronautics and Space Administration in cooperating (see *Nature* **387**, 110; 1997). But the idea of shooting objects into an asteroid raised concern about possible military applications, such as knocking down satellites, that could have had implications for international treaties governing space-based weapons.

Japanese scientists want grant process reformed

[TOKYO] A high proportion of Japanese scientists disapprove of the way funding proposals are handled by the Ministry for Education, according to a study by the Japanese Society for Biochemistry. Of 225 researchers who replied to a questionnaire sent to about 300 of the society's members, 191 agreed that the selection of grant proposals under the ministry's general university grants scheme (known as *kaken-hi*) is plagued by 'conflicts of interest'. Only seven considered the review process as 'fair'.

Scientists in the survey seem to be equally upset about the way recently established large-scale grants are awarded. And most wanted applications for the 'Research for the Future' programme of the Japan Society for the Promotion of Science to be put on an open basis (see *Nature* **383, 369; 1996).**

Nobel winner to set up Canadian gene centre

[MONTREAL] Canada's Nobel prizewinning chemist Michael Smith is trying to lure back Canadian scientists working on gene sequencing abroad by setting up a centre in British Columbia. It will be the first of its kind in Canada, researching the genes of people with breast, prostate, colon and lung cancer. The centre's scientists will try to correlate genetic information with the clinical progression of the disease.

"Canada would have been left behind if we were not getting into this field," says Smith, who shared the Nobel prize for chemistry in 1993. The new centre, to be funded primarily by the British Columbia Cancer Foundation, and other private and government agencies, will not duplicate work in centres elsewhere in the world. It opens next year in Vancouver.

Commercial whaling in return for sanctuaries?

[LONDON] The International Whaling Commission is considering a proposal from the government of Ireland for a limited reintroduction of commercial whaling in

coastal waters. The scheme was proposed at the commission's 49th annual meeting this week in Monaco. It would replace the current moratorium on commercial whaling, which is not recognized by Japan and Norway.

In return for ending the moratorium, Ireland suggests the setting-up of a global whale sanctuary outside coastal belts. But the proposal is opposed by environmentalist groups, who want the moratorium to be replaced by a permanent ban on whaling.

Population research in South Africa

[LONDON] A centre for population research is to be set up in Durban, South Africa, with the help of a £5-million (US\$8-million) grant from the UK Wellcome Trust. The African Centre for Research in Population Studies and Reproductive Health will be managed by a consortium of the universities of Natal and Durban-Westville, and the South African Medical Research Council.

The centre will concentrate on a study of the 75,000-strong population of the Hlabisa district in South Africa's KwaZulu-Natal province. Projects include a study of urinary tract infections and syphilis in pregnancy, and migration and spread of HIV.

Elsevier takes over current science titles

[LONDON] Elsevier Science, publishers of the *Trends* group of journals, have taken over several companies in the Current Science Group (CSG), including the electronic information services BioMedNet and ChemWeb. Elsevier will also acquire *Current Biology* and *Current Chemistry*, as well as the *Current Opinion* journal series.

But other titles, such as *Science Archive*, *Science Press*, *Current Medicine* and *Current Drugs*, will remain within the CSG fold. Elsevier has already been involved with both BioMedNet and ChemWeb, which were launched by CSG as a joint venture with MDL Information Systems Inc., a US-based subsidiary of Elsevier Science. The new move took place shortly after the announcement that Reed Elsevier, the parent company of Elsevier Science and the world's largest scientific and professional publishers, is to merge with the Dutch publishing company Wolters Kluwer.

Erratum: Axel Kahn

Contrary to the headline on a news item in last week's *Nature* (see *Nature* **389**, 656; 1997), the medical geneticist Axel Kahn is not leaving the Cochin Institute of Molecular Genetics in Paris when he takes up his new position as deputy scientific director for health and agricultural biotechnology at Rhône-Poulenc. Although he will devote half his time to his new post, he will maintain his research activities at the institute. Kahn is also head of an INSERM unit at the institute, and not, as the article said, director of the institute itself. We apologize for the errors.

How to make Kyoto a success

Sir — In Kyoto in December, the Framework Convention on Climate Change (FCCC) is expected to be strengthened. Negotiations have focused on commitments to cut carbon dioxide (CO₂), methane and nitrous oxide, the three main contributors to global warming.

Two other actions could make Kyoto more successful¹. One would yield quick results with benefits for thousands of generations. The other will make it possible to implement deep cuts in emissions of the main greenhouse gases in the future, if that proves necessary.

First, delegates in Kyoto should adopt strict limits on emissions of long-lived industrial gases^{1,2}. Particularly important are sulphur hexafluoride (SF₆), an insulator in heavy electrical equipment, and the perfluorocarbons (PFCs) tetrafluoromethane (CF₄) and hexafluoroethane (C₂F₆), released mainly during industrial production of aluminium and semiconductors.

Although CO₂ has caused 170 times as much global warming, the long lifetimes of SF₆ and the two PFCs — respectively perhaps 3,200, 50,000 and 10,000 years — make them a near-permanent legacy of the industrial era. Even as their concentrations rise at up to 7 per cent per year, the full range of their atmospheric effects remains poorly understood yet practically irreversible.

At best, the likely Kyoto agreement will include long-lived gases along with the three main greenhouse gases. Appealing in principle, this 'comprehensive' approach will probably encounter severe barriers — in reaching political agreement, establishing monitoring regimes and adopting global warming potentials — that

would wrongly delay the regulation of SF₆ and PFCs.

A separate agreement to eliminate these gases would be better. Voluntary regulatory programmes in Germany, Norway and the United States show the large reductions that are possible — in those countries, aluminium producers are cutting PFC emissions by half in only a few years.

A stringent international agreement would cut emissions further and worldwide. Using the effective agreement to eliminate substances that deplete the ozone layer as a model, a ban on SF₆ and PFCs could include exemptions for 'essential' emissions so that regulation does not exceed the availability of substitute technologies and processes. Periodic expert reviews could ensure that exemptions are genuine.

Second, FCCC negotiations have been plagued by a large gap between proposals for new commitments and available information on what countries can actually implement. Kyoto can narrow the gap with a nonbinding agreement for countries to develop and review 'implementing legislation' — the laws, regulations and policies they must adopt to put international commitments into practice.

More attention to implementation would improve the capacity to negotiate realistic commitments in the future. Especially if the main greenhouse gases are cut steeply, countries will be reluctant to implement costly policies unless they can see their economic competitors preparing to impose similarly expensive measures.

Another benefit would be a shift in the style of negotiating FCCC commitments by requiring that implementation plans are the

basis for negotiating new commitments. As an antidote to the plethora of unrealistic proposals that have marked FCCC negotiations so far, countries and interest groups that favour stricter controls would be expected to show how new limits can be put into practice.

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1. Victor, D. G. & MacDonald, G. J. *Linkages*

(<http://www.iisd/Linkages/Journal>, forthcoming).

2. Cook, E. *Lifetime Commitments: Why Climate Policy-Makers Can't Afford to Overlook Fully Fluorinated Compounds* (World Resources Institute, Washington, 1995).

Sir — It is not so much mandatory targets for greenhouse gas emissions reduction that are the problem (*Nature* **389**, 429; 1997) but targets that are unrealistic within the timetables proposed (2005 and 2010).

This is partly because there is confusion between the technical potential for emissions reduction and what is socially and politically feasible, given the current attitudes and behaviour of most end-consumers of energy, and the lead times involved in providing non-fossil-fuel alternatives.

As the World Energy Council has indicated in its statement for the forthcoming Kyoto conference, there is huge potential for change over the next century. But we should not be unrealistic about what can be achieved in 8 or 13 years.

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All sorts of authorship

Sir — Christopher Stubbs¹ claims that science is best served by alphabetical ordering of authors. Is this really the only alternative? Why not rotating alphabetical authorship and reverse alphabetical order to alleviate 'alphabetic disorder'²?

The earlier letter on surnames by Mark Shevlin³ reminds me of a well-known British professor's first encounter with the *Genetics Citation Index* in 1963. He could not comprehend why his name did not appear in it. Because he usually deferred to his junior colleagues by placing his name last on bylines, his name did not turn up. Later, when we launched the *Science Citation Index* in 1964, his name was included in the *Source Index* where all

authors' names are included and cross-referenced to the first author.

First authorship is mistakenly considered essential if one is to be fully credited in the citation game. But any informed citation analyst relies on an author's full CV to determine the first author of the papers to be included in the citation count.

The first comprehensive 'all-author' study did not occur until 1978 (ref. 4). Previously, 'first-author' studies were the norm. Nevertheless, many administrators persist in using the first-author data found in the printed *Science Citation Index*, disregarding its potential bias towards second authors. *The Web of Science*, an expanded version of the *SCI*, overcomes this problem because all authors' names,

thanks to hyperlinks, are displayed in the *Citation Index* section.

But what about the absurd 'no-author' policy for leading articles published in *Nature* and other journals? Is this fiction designed to impress readers with the journal's authority?⁵ Are these editorials written by robots or people named 'anonymous'?

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1. Stubbs, C. *Nature* **388**, 320 (1997).

2. Garfield, E. *Current Contents* **1**, 5–6 (1971).

3. Shevlin, M. *Nature* **388**, 14 (1997).

4. Garfield, E. *Current Contents* **28**, 5–17 (1978). Reprinted in *Essays of an Information Scientist* **3**, 538 (1980).

5. Garfield, E. *Current Contents* **9**, 5–7 (1976).

Pest adaptation

Sir — The News item about the “Dispute over insect resistance to crops” (*Nature* **388**, 817; 1997) focuses attention on the critical issue of pest adaptation to crops genetically engineered to express insecticidal proteins from the soil bacterium *Bacillus thuringiensis* (Bt). The article appropriately conveys the message that the knowledge required to design effective, scientifically based resistance management plans is not at present available.

However, the article’s emphasis on conflict between academic scientists and industry is somewhat misleading, because it is widely recognized that the urgently needed progress will come only with cooperation, rather than confrontation. I am characterized in the article as “one of UCS’s [Union of Concerned Scientists’] scientific advisers”.

In fact, like other academic scientists, I have expressed my views in publications available to all concerned parties (see *Proc. Natl Acad. Sci. USA* **94**, 3488–3490; 1997) and have provided, upon request, specific information to industry and to the Environmental Protection Agency, as well as to organizations such as UCS. Like other academic scientists, I have concentrated

primarily on generating and disseminating knowledge about pest resistance that is essential for improving management strategies. As we struggle together to create management plans based on the scant data available today, the necessity of committing public and private resources to improving understanding of resistance becomes ever clearer.

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Value for money in US laboratories

Sir — Frederick Sachs argues that the number of US National Institutes of Health (NIH) grants should be limited to two per investigator in order to fund additional young investigators (*Nature* **388**, 222; 1997). Although there may be merits to limiting further funding to investigators with a large number of grants or resources, the suggested limit would be too restrictive.

Such sums would support only a relatively small laboratory. Clearly the best qualified senior scientists can and should be able to support significantly larger and

more productive research laboratories. Unfortunately, Sachs fails to provide proof of his assertion that shifting funds from larger laboratories to a greater number of smaller laboratories would provide more major discoveries.

His discussion on Nobel prizes is irrelevant. Very few scientists will receive such an accolade and often it will be for a body of work spanning their career rather than a single discovery. This type of award system cannot recognize the excellent and critical research performed by thousands of other researchers. Indeed, major discoveries are usually built on a framework of preceding supportive work that comprises most of the funding from NIH and other agencies and which accounts for the majority of scientific literature.

The criterion of performance per existing research dollar by an investigator should be established and used to guide future funding decisions. The research dollars would include all funding available to an investigator and his or her laboratory, including research grants, endowments, awards to graduate and postdoctoral students, industrial support and donations.

Of course, any judgement of academic performance (number and impact factor of publications, for example) does include an arbitrary component, but the suggested formula is more quantifiable than existing

procedures. For example, a junior investigator with one grant totalling \$100,000 and one article in *Nature* has performed 2.5 times better than a senior investigator with grants totalling \$1 million and four papers in this journal, and should thus receive priority for an increase in funding over the senior investigator. The application by granting agencies of this formula for high-risk grants could also be useful in an era when 'safe science' grants often score better than they necessarily should.

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A probable paradox

Sir — In September 1993, in your journal, we applied Gott's formulae¹ to predict that the then Conservative UK government would, with 95% confidence, continue for a period between 4.3 months and 546 years².

In fact the Conservative government lost office 3.6 years later, so that the prediction could be regarded as confirmed³. The

success of our prediction naturally pleases us. As we see it, however, there are two problems with this minor success story.

First, had the Conservatives retained power, our prediction would still not be violated. Labour or Conservative victories would both have been in agreement with our inequality. Indeed, any electoral outcome during our lifetimes, or those of our children's children for that matter, would be in agreement with our prediction!

Second, Gott's paper¹ is now 51 months old. It has not so far been refuted (see ref. 4 for a catalogue of successful predictions). Nevertheless, experience has shown that any theory in physics, however successful, is only an approximation to reality and will eventually be refuted and require modification.

Thus, assuming the Copernican hypothesis with respect to the refutation of Gott's theory, and applying the conclusions of Gott's theory to his theory, then with 95% confidence it will remain unrefuted for at least $51/39 \sim 1.3$ months. We can regard this condition as satisfied.

In addition, however, we predict with 95% confidence that it will have been refuted after 39×51 months, that is, 165.75 years. Thus we have the paradox that, on the one hand, if the theory is refuted within the next 166 years, then its predictions will be verified. On the other

hand, if it is not refuted within the next 166 years then it does not satisfy the prediction that, with 95% confidence, it should have failed by the year 2163 and is therefore probably wrong, with 95% confidence. We are here clearly close to a Russell-type paradox, albeit in terms of probabilities.

We cannot use a 100% confidence limit in the argument, as the upper limit of permitted values then becomes infinitely large and the theory becomes meaningless. On the other hand, use of any upper confidence limit (less than 100%) makes the theory vulnerable to a Russell-type paradox. It is saved from this paradox only by the existence of this probability.

We conclude that, while the Gott argument can undoubtedly be enlightening in individual cases, as a general theory it is subject to serious shortcomings.

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Harried hen harriers

Sir — Further to Robert May's News and Views article¹ on the illegal persecution of hen harriers on grouse moors, figures can be put on the potential economic gains to the grouse-shooting industry of such persecution. They are in fact minimal.

In the same paper that May discussed, Etheridge *et al.*² calculate that 55–74 breeding harriers are removed from moors each year. The birds' main prey is usually song birds, small rodents and young hares and rabbits^{3,4}; they do take grouse chicks, but healthy breeding adults only rarely.

A hen-harrier nesting territory may encompass several hundred red grouse territories each producing, in a good year, as many as five young grouse. Were the harriers that are being illegally destroyed each year allowed to live, far fewer than 5,000 grouse chicks would escape their predation — a tiny proportion of the million or so young produced by Scotland's 250,000 breeding pairs of red grouse⁵.

Another paper⁶ indicates that many more red grouse, most of them breeding birds, are killed against deer fences each year than are taken by the hen harriers. In a survey of 135 km of deer fences, carried out over a year at 27 different sites, there were 188 red grouse collisions — and, more seriously, 37 and 36 of the bigger and rarer black grouse and capercaillie. Almost all collisions would have led to the death of the birds.

These fences are extensive. There are estimates, from the best grouse areas, of 2,000 km of deer fence in woodland and much more in moorland. They are typically 1.8 metres high, and are made partly of mesh and partly of tight horizontal wires. Most grouse that hit them are travelling at great speed as they are used to blasting their way through the twigs of trees.

In many instances the fences have been erected, with public subsidy, by the very landowners implicated in killing hen harriers. Some fences are still needed to protect young forest trees from deer, but many are old and redundant.

Putting deer into the equation, if deer numbers were reduced to a third of current levels (to about six animals to the square kilometre from 15–20), natural regeneration of forest would take off. In most areas there would still be plenty of deer for stalking; and the better cover, produced by removing the surplus of feeding deer, would provide much improved habitat for the coexistence of game birds and hen harriers.

As May points out¹, the management of moorland for grouse shooting is infinitely preferable to overgrazing by sheep or deer,

or planting conifers. It remains to be seen whether the shooting interests are able to bring themselves to obey the law and stop persecuting hen harriers, and to realize the consequences of their other management activities.

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Greenpeace and BP

Sir — A recent leading article¹ states that “BP was not only within its rights but well advised to sue Greenpeace UK for sums that might well have extinguished the organization”.

Whereas it was disturbing for BP to take such a Machiavellian approach, it is quite alarming for this approach to be actively supported by *Nature*. It concurs with BP's stated objective at the time — not the recouping of financial damages from Greenpeace's occupation of the Stena Dee oil platform but the prevention of Greenpeace's campaigning efforts, allowing BP to continue oil exploration on the Atlantic frontier.

Your applause for BP fails to recognize BP's contribution to the climate change problem, Greenpeace's campaign or our continuing ‘constructive engagement’ with oil companies and the climate debate.

As you point out, BP has at last to some degree distanced itself from what has, over the past decade, been a marauding pack of oil companies determined to destroy the credibility of climate change science and to attack any scientists who supported efforts to deal with the problem. BP's conversion has, however, so far been limited to a cautious few words, whereas its actions contradict even these first few steps.

BP leads the oil industry in areas including the Atlantic and the Arctic in finding and developing new resources of oil. BP's actions, far from dealing with the climate change problem, seem likely to extend the life of the fossil-fuel age for decades, at a time when we should be deciding to leave fossil fuels in the ground and working towards that end.

In relation to BP's solar business, you

state that BP has “shown strong support for photovoltaic [PV] energy research”. A recent study² coordinated by BP Solar found that the building of a £350-million 500-MW factory to produce solar panels would reduce solar costs by 80 per cent, making solar competitive with electricity supplied by fossil fuels.

Commenting on the BP report, Paul Maycock, editor of *PV News*, was quoted in the August 1997 edition as saying that “at these costs solar will be fully economic throughout the world”. And Allen Barnett, president of Astropower, a US solar manufacturer, has indicated that reduction in solar costs to the level shown in the BP study would generate an annual global market of \$100 billion.

It is not research into PVs, therefore, that is now required from BP but rather a commitment to expand an established solar market. In this case the commitment requires about half the investment that the Foinaven oilfield has so far cost BP.

From our attendance at every United Nations climate negotiation to our production of Jeremy Leggett's ground-breaking book on global warming in 1990, as well as our climate impact research trips to the Antarctic and the Arctic this year, Greenpeace has been “constructively engaged” in the climate change debate from the beginning.

So while climate negotiations continue to proceed in fits and starts, part of this engagement for Greenpeace must also be taking direct action to try to prevent the causes of the problem.

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Natural selection and the sex ratio

Sir — The invariable attribution¹ to R. A. Fisher of the famous argument about how natural selection controls the sex ratio is not correct. Darwin gave it in the first edition of *The Descent of Man*² and, although he withdrew it for the second edition (which was the one Fisher owned), it nevertheless found its way into the literature.

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Testing the nuclear test-ban treaty

A recent earthquake near a former Soviet nuclear test site has tested mechanisms for monitoring the test-ban treaty. Technical systems passed with flying colours, but relevant US agencies could have done better.

Paul G. Richards and Won-Young Kim

Early on the morning of Saturday 16 August 1997, there was a small seismic event in the Kara Sea, about 100 kilometres from the nuclear test site on the far northern island of Novaya Zemlya now used by Russia for nuclear weapons research (Fig. 1). Within days, seismologists located the event in an area where it could only have been an earthquake.

Yet at the end of August, spokesmen for the US State and Defense Departments described the event as having “explosive characteristics”, and in late September officials of other US agencies were still characterizing it as “unresolved” (George Ullrich, Defense Special Weapons Agency) or “lending itself to alternative interpretations” (Bob Bell, National Security Council).

These statements suggest that the seismic signals might have been generated not by an earthquake but by a nuclear test. Underground nuclear explosions were carried out by the Soviet Union at the Novaya Zemlya test site from 1964 to 1990. But Russia and many other countries signed the Comprehensive Test Ban Treaty (CTBT) in September 1996, so making a commitment not to carry out nuclear explosive tests for any purpose.

To a seismologist, the evidence is straightforward: the event took place several tens of kilometres offshore to the southeast of Novaya Zemlya, in an area where water depths are around 400 metres. Nuclear explosive testing is not credible in such an ocean environment unless there is other evidence, such as signals from hydroacoustic or radionuclide detectors, or the presence of a vast and complicated drilling operation. The event must therefore have been an earthquake.

On 23 September, President Bill Clinton submitted the CTBT to the US Senate for advice and consent to ratification. The 16 August event and its aftermath will provide valuable input to the discussion surrounding the Senate's deliberations, including the question of whether the treaty is verifiable. As we show here, analysis of this small earthquake indicates excellent capability to monitor the Russian nuclear test site.

But, paradoxically, the event is being used in some quarters to cast doubt on the verifiability of the CTBT¹. The unfortunate lack of clarity in the handling of this ‘problem event’ — of which there will be many more in years to come — shows that the United States must develop the necessary government forums to evaluate relevant technical inputs and summarize them appropriately for policy-makers.

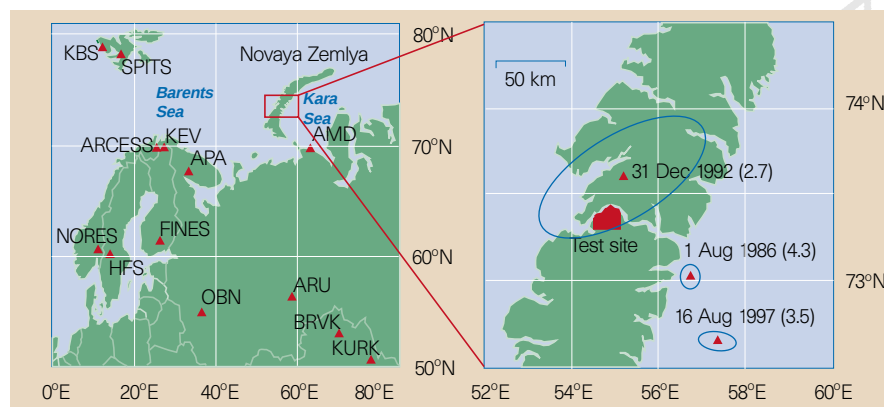


Figure 1 Maps showing the Novaya Zemlya test site, locations and magnitudes of recent earthquakes, the 16 August event and some of the relevant seismographic stations. Earthquake locations and their uncertainty are characterized by error ellipses²⁻⁴, which give 90 per cent confidence intervals.

When the CTBT comes into force, an international data centre in Vienna will receive data from an international treaty monitoring system (IMS) and process them to provide basic information such as the locations of sources of various signals. A prototype international data centre (PIDC) in Arlington, Virginia, has begun the work of CTBT monitoring, receiving data from global networks of hydroacoustic, infrasound and radionuclide sensors (as yet incomplete) and seismic data from stations of the IMS (essentially complete for coverage of Novaya Zemlya).

It is not the responsibility of the PIDC to determine whether detected events are explosions or earthquakes, but rather to provide the data to national data centres of treaty signatories so they can make their own determinations. The 16 August event provides an opportunity to assess the performance of national and international data centres, and the contribution of stations outside any treaty-monitoring network.

How monitoring worked in practice

Although the nearest IMS station to the event, an array of sensors at ARCESS in northern Norway, was (unusually and unfortunately) not operating on 16 August, the PIDC nevertheless received adequate seismic data to detect and locate the event automatically that morning, about an hour after it happened. The event was studied by PIDC experts a few days later. Their best estimate of the location, published in their *Reviewed Event Bulletin (REB)*², is quite close to the automatically determined location. Automatic locations, not always reliable, are routinely available to national data centres and are important indicators of what data are available at the PIDC. Figure 1 shows the

REB error ellipse as well as the location. Such error ellipses “must be taken as only tentative indicators of the actual epicentral location accuracy”³, but a key point for the event of interest is that different error ellipses based on different choices of Earth structure all lie in the Kara Sea and do not overlap the land. The REB location (Fig. 1) is consistent with data from other stations that have subsequently become available, including some from as far away as Kazakhstan.

Of course, there will be some earthquakes on the land area of Novaya Zemlya, and, indeed, near other countries’ test sites — on 12 September a magnitude 4 earthquake took place directly under the US nuclear test site in Nevada. For such events, a way other than location is needed to discriminate between earthquakes and explosions.

For small seismic events at Novaya Zemlya, a systematic difference between earthquakes and explosions can be seen, if signal-to-noise ratios are adequate, in the high-frequency seismic waveforms for compressional waves (P waves) and shear waves (S waves). Previous experience with the IMS station at ARCESS has indicated that this station records waveforms extending well above 10 Hz for earthquakes and explosions at Novaya Zemlya, and that the strength of such high-frequency signals is indeed systematically different for the two types of seismic source, the P/S spectral ratio being weaker for earthquakes than for explosions.

Stations more distant than ARCESS may receive good signals, but not at frequencies high enough for waveform-based discrimination of small events. With no ARCESS data for the 16 August event, and with no other IMS station both near enough to record high-frequency signals and having an

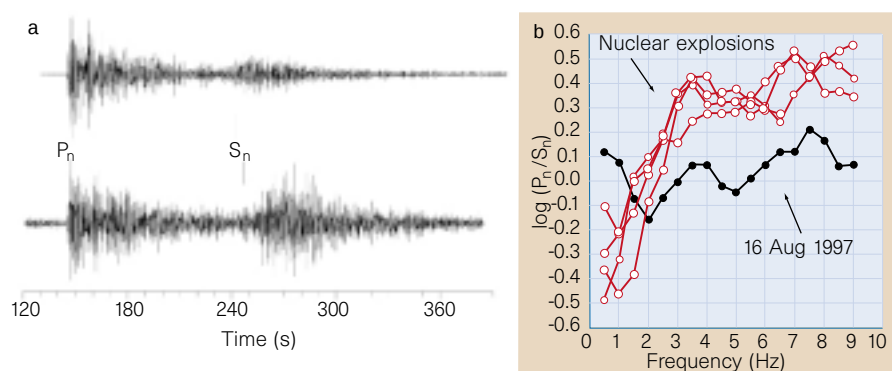


Figure 2 a, Waveforms for a Soviet-era nuclear explosion at the Novaya Zemlya test site (top, 24 October 1990, 1,048 km) and for the 16 August event (bottom, 1,127 km), recorded at the same station, KEV, in northern Finland. b, Spectral ratios for a few nuclear explosions, and for the 16 August event, at KEV. Although no earthquake records from KEV were available for reproduction here, the weaker P/S ratio of the 16 August event is a well documented characteristic of earthquakes^{3,5}. KEV is a station in the global network operated jointly by the USGS and the IRIS consortium of US academic institutions.

archive of previous Novaya Zemlya nuclear explosions for comparison, many seismologists have been working to find non-IMS stations with data suitable for discrimination based on the P/S ratio. One such station is KEV in northern Finland, not far from ARCESS (Fig. 1).

Figure 2 shows a comparison of the KEV seismograms for the 16 August earthquake and for a nuclear explosion, together with the P/S spectral ratios for explosions and for the event of interest. These data show the distinctive feature of a weak (earthquake-like) P/S ratio for the 16 August event. Incidentally, that the delay between P- and S-wave arrivals is longer (by about eight seconds) for the 16 August event than for the nuclear explosion is in itself clear evidence that the event could not have happened at the nuclear test site.

Several other lines of evidence support identification as an earthquake. For example, there was an aftershock nearby about four hours after the main event. Aftershocks are common for earthquakes, but would not be detectable after small nuclear explosions at Novaya Zemlya. Comparison of the main-shock waveforms with those from an earthquake on 1 August 1986 also shows that the 16 August event was an earthquake. Further studies are under way to estimate its depth, which can provide yet another discriminant.

Implications

The ease of location and identification of the 16 August event, with a magnitude of about 3.5, demonstrates that the CTBT can be monitored near the Russian test site down to magnitude 3, and maybe even lower. Magnitude 3 translates to about a twentieth of a kilotonne for a nuclear explosion that is well coupled to the ground, and a few kilotonnes for a fully decoupled test. (To decouple the explosion from the surrounding rock requires construction of an underground cavity of more than 100,000 m³ and even

then the explosion would generate signals that could provide the basis for an on-site inspection request.) So the 16 August event indicates excellent capability to monitor the CTBT.

But this straightforward implication for the treaty is being obscured by the fact that the 16 August event is still “unresolved” by US officials, who are now in the unenviable position of having to accept the earthquake interpretation (critics will ask why it took so long) or of continuing to argue for alternative interpretations (critics will ask for evidence, and for reasons why the obvious interpretation should not be accepted). It may be difficult for these officials to accept that data from non-IMS stations and data analysis by independent groups of seismologists have had such prominence. But there are more than 10,000 seismographic stations around the world for the general purpose of research into earthquake hazard and the structure of the Earth’s interior, so supplementary data will often be available.

Unfortunately, permission was withdrawn (having earlier been granted by the Pentagon) for the PIDC director to present the basic IMS data and data analysis on 25 September to the annual CTBT research and development (R&D) symposium, this year organized by the Defense Special Weapons Agency. The meeting attracts about 200 people from US agencies and national laboratories, and from universities and other research contractors, as well as several participants from outside the United States. A presentation of the PIDC’s material would have widened appreciation that the international data centre is performing well.

A vigorous R&D programme in support of CTBT monitoring is essential to future improvements; and US researchers, who have recently seen the destruction of two air force programmes that long supported academics and private contractors to improve CTBT monitoring, are apprehensive about

Department of Defense plans for reorganization in this area. An R&D programme that subordinates problem-solving to bureaucratic concerns — which probably lay behind the withdrawal of permission for the PIDC director to speak at the recent symposium — will drive leading researchers from the field.

When the research community can demonstrate a good new method of discrimination, or the need for good communication to non-IMS stations with openly available data, the development must be assessed and operational procedures perhaps revised. Regrettably, there can be great resistance to such revisions — in the United States, after research results in the 1970s pointed to a seismic magnitude bias between Soviet and US test sites, it took more than 10 years of wrangling to change government procedures for estimating the yield of Soviet explosions.

Practical issues of national data centre operation, and how to evaluate suspicious events, are likely to arise soon in debates on organization of the US National Authority for the CTBT — which will have to be established by new domestic legislation to do the real work of advising US policy-makers on technical issues of CTBT verification.

Encouragingly, an agreement has recently been reached between the Department of Defense and the US Geological Survey (USGS) on the role of the USGS in generating and circulating IMS data. The USGS is to have “timely access to all CTBT IMS data... relevant to USGS programs and missions” — which may be the way these data are made accessible to researchers in nongovernmental organizations working to improve methods of discrimination. These researchers have been handicapped in studying the 16 August event and its aftershock because many have been unable to obtain relevant IMS data. The USGS of course has expertise in studying earthquakes, and has a long tradition of working to make data openly available. It remains to be seen if limits are somehow still put on distribution of IMS data.

It is truly a difficult management problem to handle treaty monitoring where key technical skills often lie outside government agencies, and where rapid decisions may be needed on problem events but the best data may come in days or weeks later. The event of 16 August has provided an opportunity to show what can go wrong, and what can go right. □

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Some like it hot: spicing up ion channels

David E. Clapham

Capsaicin is the molecule that makes chilli peppers seem hot, and the receptor for this compound has now been identified. The receptor is also activated by increased temperature, indicating that it transduces painful thermal stimuli *in vivo*.

I bit into the little piece of red vegetable, mischievously hidden under the grains of rice, and a fire exploded in my mouth. Instinctively, I put my hand over my mouth and unwittingly spread the fire to my eye. I then tried to douse the fire with water, only to have it spread around my whole mouth. I broke into a sweat ... This sequence of petty disasters is repeated daily by hot-food novices eating at Mexican, Indian, Chinese, Indonesian and other restaurants serving cuisine from the tropical belt. Two questions come to mind. First, how do hot spices do this to our nervous system? Second, why do we do this to ourselves (after all, the menu said it was *hot*)?

The first question is answered on page 816 of this issue¹, with the molecular identification of an ion channel that is responsible for the response to peppers. Members of David Julius' laboratory have identified the protein that comprises the receptor for capsaicin and painful heat sensation. Capsaicin is found in the white 'ribs' inside hot chilli peppers, and it elicits the familiar sense of burning pain. It is just one molecule in the chemical-warfare armoury of plants and, presumably, it was designed to protect plants from being eaten (humans, of course, ignore this polite warning).

Capsaicin is so potent that its effects are calibrated by the spice industry in a sort of taste Richter scale (Scoville heat units), ranging from Bell peppers (<1), jalapeño (10³) and habanero (10⁵), to pure capsaicin (10⁷). In 1912, Wilbur Scoville calibrated the potency of pepper by extracting capsaicin in alcohol and diluting it until pungency was just detected after placing a drop on his tongue². Although Dr Scoville is no longer around to assign heat units, the scale is still a subjective psychophysical one.

Using an innovative expression-cloning strategy, Julius and co-workers have isolated the complementary DNA encoding the protein that binds capsaicin. Based on other studies^{3,4}, they knew that capsaicin activates an ion channel that allows calcium ions to flow from the sera (where they are found at

high concentrations; 2 mM) into the intracellular space (where the concentration is around 100 nM). This change can be detected by calcium indicator dyes — molecules that can be introduced into a single cell, and shift their fluorescence emission spectra on chelating calcium⁵.

The receptor protein was known to occur in neuronal cells, the fibres of which carry pain from the periphery (such as the tongue) to the dorsal roots of the spinal cord. Taking messenger RNA from dorsal root ganglion cells, the authors constructed a 'library' of the cDNAs from which all of the expressed proteins in these cells are made. The library was then divided into large groups which were put into cells. By weeding out the single cDNA that made the cells respond with a fluorescent flash after exposure to capsaicin, they determined the DNA-encoded amino-acid sequence of the protein that comprises the capsaicin receptor. Because the vanilloid group chemically defines capsaicin, the receptor was dubbed the vanilloid receptor subtype 1, or VR1. But a better name might have been the HOT channel.

Once a cDNA is identified, it is simple to engineer immortalized cells to make the protein and incorporate it into their plasma membranes. The expressed capsaicin receptor shows the same properties as capsaicin receptors in sensory neurons: it is a relatively calcium-selective ion channel with a large single-channel conductance. Whereas capsaicin and resiniferatoxin (from the flowering cactus *Euphorbia resinifera*) activate the current, a synthetic antagonist, capsazepine⁶, blocks its activation.

If capsaicin acts directly on VR1, the apparent cooperativity of binding suggests that there is more than one binding site. A reasonable hypothesis is that the channel pore is formed by four identical subunits, like many voltage-activated potassium-selective ion channels. Interestingly, capsaicin is lipophilic and might bind the channel from either the exterior or interior of the cell. Its affinity for fat also explains why drinking more water after a spicy mouthful

spreads the fire around, whereas absorbing the spice with food is more effective.

The VR1 channel has fascinating biological properties — it is not simply a nonselective cationic pore. Rather, like the NMDA (*N*-methyl-D-aspartate)-type glutamate-receptor channel, it passes about ten calcium ions for every sodium ion (Fig. 1). Under physiological conditions, opening of the channel will not only initiate nerve impulses by depolarizing the membrane potential, but it will also raise levels of intracellular calcium considerably. Herein lies an interesting mystery. The cell lines made to express VR1, as well as neuronal pain fibres *in vivo*, die after several hours of continuous exposure to capsaicin^{1,3,7}. Cell death seems to be necrotic (caused by injury) rather than programmed, and the authors speculate that it is induced by the continuous influx of ions.

One obvious question is whether the death of cells in the pain fibres is a natural adaptive response. Calcium-induced cell death is thought to underlie neuronal remodelling in the brain through, for example, activation of glutamate-responsive channels. Perhaps desensitization to hot

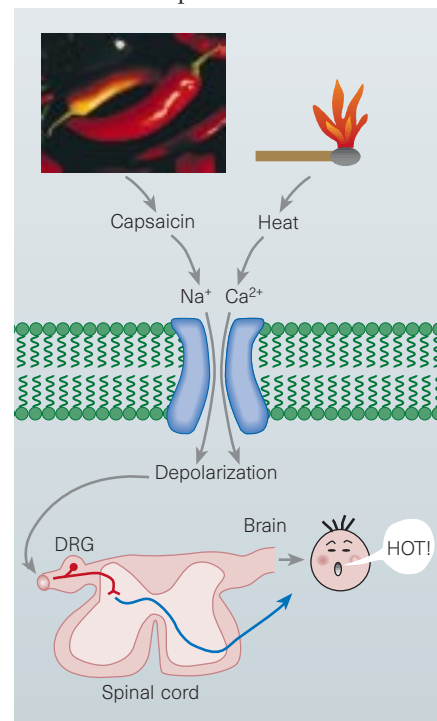


Figure 1 Hot peppers and painful heat both activate sensory nerve fibres through an ion channel discovered by David Julius and colleagues¹. The channel, known as vanilloid receptor subtype 1 (VR1), is activated by binding of capsaicin — the compound that makes chilli peppers hot. When activated, the channel opens, allowing an influx of calcium and sodium ions. The influx depolarizes neuronal pain fibres, initiating a nerve impulse through the dorsal root ganglion (DRG) to the brain. Noxious temperature uses the same elements, explaining why our mouths feel hot when we eat chilli peppers.



100 YEARS AGO

Intentional introductions of wild species, however, have almost without exception resulted disastrously In 1872 Mr. W. Bancroft Espeut imported four pairs of the Indian mongoose from Calcutta into Jamaica for the purpose of destroying the "cane-piece rat." Ten years later it was estimated that the saving to the colony through the work of this animal amounted to 100,000*l.* annually. Then came a sudden change in the aspect of affairs. It was found that the mongoose destroyed all ground-nesting birds, and that the poultry as well as the insectivorous reptiles and batrachians of the island were being exterminated by it. Injurious insects increased in consequence a thousand-fold; the temporary benefits of the introduction were speedily wiped away, and the mongoose became a pest. Domestic animals, including young pigs, kids, lambs, newly-dropped calves, puppies and kittens, were destroyed by it, while it also ate ripe bananas, pine-apples, young corn, avocado pears, sweet potatoes, cocoas, yams, peas, sugar-cane, meat, and salt provisions and fish. Now, we are told, nature has made another effort to restore the balance. With the increase of insects, due to the destruction by the mongooses of their destroyers, has come an increase of ticks, which are destroying the mongoose, and all Jamaicans rejoice. From *Nature* 21 October 1897.

50 YEARS AGO

A project for a Central Library of the World, drawn up during the German occupation of France, with the dual objects of providing more effectively for the preservation of the documents on which human culture rests and making their utilization easier and more widespread, is of interest in relation to the programme of work now contemplated by the United Nations Educational, Scientific and Cultural Organization. ... The preliminary scheme summarized in this pamphlet contemplates the establishment of the organisation, to be called the Bibliothèque Centrale du Monde, by a statute guaranteeing the absolute inviolability of the headquarters of the organisation, which is to be regarded as a world reserve; the territory where it is located should belong to no nation.... From *Nature* 25 October 1947.

foods by spice lovers is due to death of neuronal pain fibres. Alternatively, the entry of calcium may initiate intracellular regulatory mechanisms (such as phosphorylation of target proteins or transcription) that reduce the cellular response. In any case, capsaicin is known to have analgesic effects against arthritis and post-herpetic neuralgias, and desensitization of the nerve response may be the main mechanism by which it acts.

Rapid increase of temperature to levels that produce pain (noxious heat) is also thought to be sensed by the capsaicin receptors in neurons⁸. Interestingly, the VR1 channel also senses temperature at levels that produce pain, providing a plausible molecular explanation for why we perceive foods that contain capsaicin as hot. Moreover, Julius and colleagues found that rapid increases in temperature — from 22 °C to 48 °C — evoke ion currents from expressed VR1 channels that closely mimic those induced by capsaicin.

The amino-acid sequence of VR1 is most closely related to the transient receptor potential (TRP) class of proteins (reviewed in ref. 9), several subtypes of which have now been cloned in humans. There has been much talk — but no definitive data — to suggest that TRP proteins comprise the class of channels that are activated by depletion of calcium from the intracellular calcium repository. Such 'store-operated' channels might not only replenish intracellular calcium, but they may have other functions. The message that signals the plasma membrane to let in more calcium remains a mystery. Julius and co-workers rule out a store-operated role for VR1 channels: thapsigargin, which stimulates depletion of intracellular

calcium by blocking Ca-ATPase pumps, does not activate expressed VR1 channels. Moreover, most of the sequence homology to TRP proteins is in the amino-terminal ankyrin repeats, which probably act to localize and anchor such proteins.

Cloning of the noxious-temperature-sensitive capsaicin receptor adds an important member to the list of relatively nonselective ion channels that are gated by ligands. Unlike the purinergic- and glutamate-receptor-channels, the natural ligand — if one exists — is not known. It is possible that the real purpose of the channel is to sense noxious temperature, and that capsaicin is nature's low-energy way of harnessing the sensor. But it would be interesting if there turned out to be related channels and, perhaps, intrinsic agonists that could be used as pharmacological targets. Certainly, clinical management of chronic pain — such as that caused by arthritis, spinal-cord injury or diabetic neuropathy — could use new therapeutic strategies. At the very least, Mexican restaurants might be able to provide antidotes for their jalapeño-challenged clientele. □

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Sensors

New age crystals

David G. Grier

How much lead is in your drinking water? How much glucose is in your bloodstream? Assaying trace components in complex solutions usually requires the sophisticated facilities of an analytical laboratory. But a new class of composite materials created by Holtz and Asher at the University of Pittsburgh may revolutionize such measurements, moving them out of the laboratory and into everyday life¹. The sensors they describe on page 829 of this issue combine sensitivity with specificity in an economical system that is appropriate to a very wide range of applications. Moreover, the sensors are strikingly beautiful, shimmering with the colours of the rainbow (Fig. 1).

The active elements in the new sensors are chemically functionalized gels. These gels are networks of crosslinked polymer strands, saturated like a sponge with a fluid solvent.

Small changes in the fluid's composition can lead to dramatic changes in the gel's volume through a combination of processes known collectively as swelling. The degree to which a gel swells is controlled by a delicate balance of interactions between the polymer and its solvent, and between different chemical domains on the polymer itself. Changing the balance, for example by changing the gel's temperature, causes the polymer network to expand or contract.

Chemically functionalized gels can be tailor-made to respond strongly to a specific stimulus, such as the concentration of a particular ion in solution. The trick is to incorporate chemical groups into the polymer whose response to the desired stimulus induces a swelling transition. The functional groups highlighted in Holtz and Asher's study respond to specific chemical species by

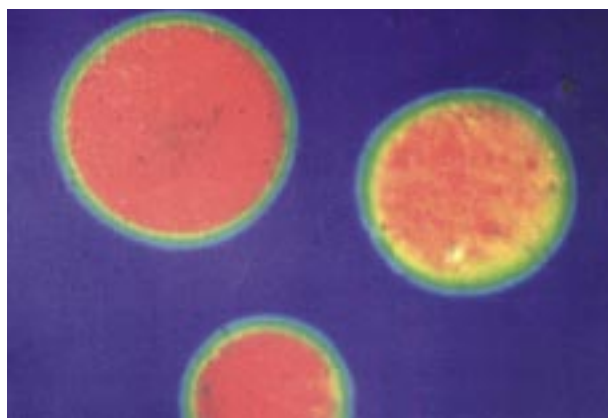


Figure 1 **Leaded lights:** a new gel sensor shows three drops of dilute Pb^{2+} solution, spreading slowly. A chemically functionalized gel fills the space between tiny plastic spheres that form a colloidal crystal. This usually diffracts violet light towards the angle observed here, but when the lead solution diffuses through it the gel swells, increasing the spacing of the crystal so that it diffracts red light.

developing electrostatic charges whose mutual repulsion causes the gel to swell. Other gels swell in response to light² or pH³. The response to these stimuli can be calibrated precisely and is highly reproducible.

Until now, gels' utility as sensor materials has been limited by a lack of accurate techniques to gauge their swelling. The Pittsburgh group have solved this problem by encapsulating colloidal crystals within their gels to form composite materials whose optical properties respond strongly to small chemical changes.

Colloidal crystals are regular arrays of small particles, often spheres, suspended in a fluid⁴. Hard spheres experiencing only contact repulsion form rigidly ordered crystals when they fill roughly half of the available volume; charged spheres with longer-range interactions crystallize at much lower concentrations. These crystals have lattice constants that are comparable to the wavelength of light, and so they act like three-dimensional diffraction gratings, resolving incident white light into its component wavelengths. The resulting blaze of pure colours is familiar from peacock feathers, butterfly wings and gem opals, all of which owe their beauty to such ordered microstructures. Changing a crystal's lattice constant changes what colour it scatters into a particular angle, and the Pittsburgh sensors exploit this effect to measure swelling.

Creating a functionalized gel around a colloidal crystal is not much more complicated than adding fruit to a gelatin dessert. Colloidal spheres can be dispersed in a solution of reagents used to make a functionalized gel. Once the spheres have assembled themselves into a crystal, the gel can be polymerized around it. Individual spheres either become chemically bound to the polymer network, or else become tangled within the web. Then, as the gel swells, the crystal's lattice constant changes by an amount that can be measured optically.

A practical sensor might consist of a small chunk of composite gel, probably smaller than a cubic millimetre, attached to the end of an optical fibre. This end would be dipped into a test solution whose constituents would diffuse into the gel, coming

into equilibrium with the chemically-specific binding sites. A spectrophotometer at the dry end of the fibre would then gauge the gel's response to its target stimulus by measuring its optical reflectivity.

Different gels would be needed to measure different stimuli. The necessary volume of gel is so small, however, that each optical probe should be very cheap. Moreover, because gels can be sensitized to a wide range of stimuli, many different tests could take advantage of the same optical read-out equipment. Indeed, an array of such sensors could be devised to perform highly efficient combinatorial assays along the lines pioneered by Peter Schultz and co-workers at Berkeley⁵. This flexibility and generality contrasts with conventional techniques, such as conductivity probes for pH and ionic concentration, which tend to be highly

specialized, delicate and expensive.

In addition to their practical applications, gelled colloidal crystals are likely to be interesting in their own right. For instance, they might help to resolve a heated controversy regarding interactions between charged colloidal spheres^{6,7}. Experiments and theoretical considerations imply that within a colloidal crystal, like-charged spheres may attract one another, contrary to conventional understanding. Optically measured structural changes in gelled crystals might provide new insights into the anomalous attraction's mechanism. Conversely, the spheres might serve as useful tracers⁸ with which to probe the structure and dynamics of the gels, neither of which is particularly well understood. Both as sensors and as subjects for fundamental research, functionalized gelled colloidal crystals have a bright future. □

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Genomic imprinting

Disomy and disease resolved?

Nicholas Hastie

Why is it that babies with two doses of chromosome 11p15 from their father — instead of one from each parent — have disproportionate overgrowth of organs such as tongue, muscles, kidney, liver and heart^{1,2}? This is the so-called Beckwith-Wiedemann syndrome (BWS)³, and other symptoms include omphalocele (an abdominal-wall defect), thickening of long bones, renal dysplasia (abnormal growth), adrenal cytomegaly and polyhydramnios (excess amniotic fluid).

On page 809 of this issue, Sun *et al.*⁴ report that they have used a transgenic-mouse approach to provide convincing evidence that some, but not all, of these problems arise because the children have double the normal dose of insulin-like growth factor-2 (IGF2). The *IGF2* gene on chromosome 11p15 is remarkable in that it is imprinted — in other words, only one of the two parental copies is normally active. In this case, the paternal copy is active whereas the maternal gene is silent in most tissues. So a baby with a paternal duplication of chromo-

some 11 would be expected to have a double dose of the protein.

IGF2 has long been a front runner for a BWS factor. It is expressed at highest levels in the tissues that are most affected in BWS. Moreover, mutations in mice that lead to increased (indirectly) or decreased expression of *Igf2* result in overgrowth or undergrowth, respectively^{5,6}. Most compelling is the fact that *IGF2* imprinting is lost in 80% of BWS cases, so the maternal allele is also expressed⁷. But all of the evidence so far has been circumstantial, the problem being that there is a cluster of imprinted genes on chromosome 11, and no chromosome rearrangements or mutations that affect expression of *IGF2* alone³ have been identified in BWS patients.

To sort things out, many researchers have asked whether mice with additional functional copies of the *Igf2* gene would develop BWS symptoms. But it has been impossible to obtain transgenic mouse lines expressing increased doses of *Igf2*. So Sun *et al.*⁴ have taken an alternative route. They introduced

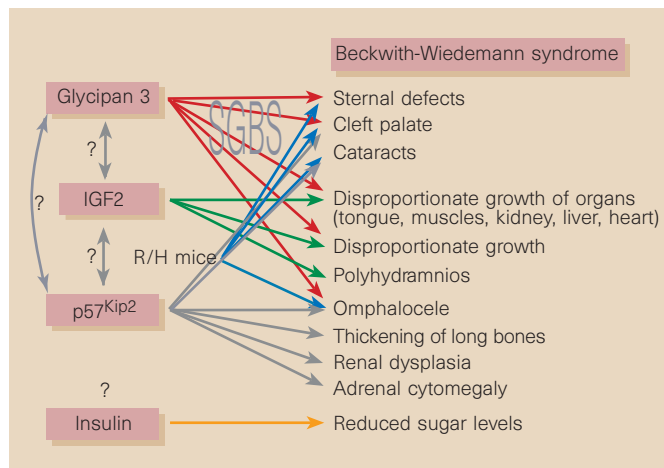


Figure 1 Clinical overlap and possible interactions between genes involved in Beckwith-Wiedemann syndrome and Simpson-Golabi-Behmel syndrome (SGBS). Note that not all possible interactions may be shown.

an extra copy of the *Igf2* gene into embryonal stem cell lines. These cells were then mixed with normal embryos to make chimaeras. The chimaeras that expressed a 1.5–3-fold increase in *Igf2* were overgrown, they had disproportionate overgrowth of the tissues that are normally affected in BWS, and polyhydramnios was associated with the pregnancy. In each case, there was an excellent correlation between the size of an organ and its level of *Igf2* expression, supporting the idea that IGF2 may work at short range in the body.

But overexpression of *IGF2* does not account for all of the BWS phenotypes — what about omphalocele, adrenal cytomegaly, bone defects, renal dysplasia and hypoglycaemia (Fig. 1)? Most of these features can probably be explained by loss of activity of a second imprinted gene that encodes the cell-cycle regulator *p57^{Kip2}*. This protein inhibits the activity of cyclin-dependent kinases^{8,9}, and it is expressed from the maternal gene copy on chromosome 11p15. Hence, paternal duplication with loss of the maternal chromosome would lead to the reduction or absence of *p57^{Kip2}* activity. Loss-of-function mutations in the *p57^{Kip2}* gene have, in fact, been described in several people with BWS^{10–12}. However, as these represent only 10% or so of cases, the general significance of this gene in BWS has been questioned.

In the case of *p57^{Kip2}*, clarity came from studying mice in which the gene was specifically disrupted^{8,9}. These mice developed omphalocele, renal dysplasia, adrenal cytomegaly and bone defects, but there was no disproportionate overgrowth or polyhydramnios. So, adding together the effects of *IGF2* overexpression and *p57^{Kip2}* loss of function, we can account for most — but not all — BWS phenotypes. Neither mouse model showed reduced sugar levels. Perhaps this is explained by overexpression of a third gene, the insulin gene, which also maps to 11p15 and may be imprinted.

Is there any evidence for communication between the pathways involving *IGF2* and *p57^{Kip2}*? Clues can be found from examina-

tion of another mouse model that has just been produced by Eggenschwiler *et al.*¹³. Using a clever combination of genetic crosses, these authors created mice (R/H mice) with up to a sevenfold increase of *Igf2* in their circulation. The R/H mice died at varying stages during fetal development and never reached term. They were up to 200% of the normal body weight and, like the mice described by Sun *et al.*⁴, they had disproportionate overgrowth of certain organs. Like the *p57^{Kip2}*-mutant mice, the R/H mice also had an omphalocele, one of the most consistent features of BWS. The two types of mutant mouse also shared three other phenotypes that are not usually considered part of BWS — cleft palate, cataracts and sternal defects. Notably, cleft palate and cataracts have now been documented in a small proportion of BWS patients, and these shared phenotypes indicate that there may be some crosstalk between the *IGF2* and *p57^{Kip2}* pathways.

These mouse models also reinforce the possible mechanistic link between BWS and another human overgrowth syndrome, the Simpson-Golabi-Behmel syndrome (SGBS)¹⁴. Children with SGBS have often been misdiagnosed as having BWS. However, SGBS is more severe, and 50% of affected babies die immediately before or after birth. Also, some of the phenotypes that are observed in SGBS are not found in BWS — particularly defects of the sternum and vertebrae. SGBS arises through mutations in the *glycican 3* gene on the X chromosome¹⁵. A tantalizing claim was that glycican 3 (which is a membrane proteoglycan) could bind IGF2, potentially reducing the effective concentration of IGF2¹⁴. This would provide a satisfying explanation for the overlap of symptoms between the two syndromes, because loss of glycican 3 function would lead to increased levels of IGF2. Although this idea lost favour when another group could not reproduce the IGF2–glycican 3 interaction¹⁶, the mouse models have again shed some light. The R/H mice showed a number of SGBS-specific patterns, particularly sternal and vertebral defects¹³. Perhaps,

after all, IGF2 and glycican 3 interact either directly or indirectly.

In science, we are always hoping for the surprise result that will take us in new directions. Sun *et al.*⁴ were the beneficiaries of such an unexpected bonus. In the chimaeric mice that carried an additional copy of the *Igf2* gene, the increased RNA and protein were not expressed from the transgenic copies but, rather, from the endogenous chromosomal copies of *Igf2*. So perhaps DNA sequences in the transgene were binding to, and sequestering, proteins that normally repress expression of the chromosomal copies.

In summary, transgenic mice have helped to unravel the mechanisms of two complex human diseases⁵. The studies also provide new insights into the way pathways intersect, and into different mechanisms of gene regulation. Moreover, the follow-up studies should tell us a lot about how *IGF2* is regulated, and even about the process of imprinting itself. □

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Avoided object

Readers who have not read the explanatory leading article (see *Nature* **389**, 213; 1997) may have been bemused by the appearance over the past five weeks, and the disappearance this week, of the series under the above title by Cornelia Parker. That series was commissioned in collaboration with the Ruskin School of Art and Design at Oxford University and sponsored by Southern Arts and the Arts Council of England. A new series of articles on art and science begins this week on page 799.

Atmospheric chemistry

A bad winter for Arctic ozone

Richard Stolarski

Ozone loss in the Antarctic, resulting in a seasonal 'ozone hole', is a familiar problem. But what of the Arctic? Why are the Northern polar regions less susceptible to ozone loss; and could that situation change? Two papers (by Müller *et al.*¹ in last week's *Nature* and Rex *et al.*² on page 835 of this issue) now report large ozone loss during the winter and early spring of 1995–96 in the Arctic. This comes after considerable losses over the previous few winters. What has been peculiar about the past few winters is that the Arctic stratosphere has stayed cold for longer than average, and this seems to have led to low ozone concentrations.

ClO concentrations in the Arctic vortex are as high as those seen in the Antarctic, more than about 1 p.p.b.v. (part per billion by volume), and when such high concentrations are exposed to sunlight, it is well established that ozone loss will take place at a high rate. It is nitric acid (HNO₃) that makes the difference: in the Antarctic, temperatures are low for long enough that particles containing nitric acid and water grow large enough to fall out of the stratosphere, leading to denitrification and dehydration. In the Arctic, temperatures are low enough for these particles to form, but not for long enough to lead to large-scale denitri-

fication or dehydration. Because there is a lot more nitric acid in the Arctic atmosphere than in the Antarctic, ozone loss occurs in competition with recovery of ClO to chlorine nitrate (ClONO₂) via reaction with NO₂ released from nitric-acid photolysis. So less ozone loss can occur here than in the Antarctic, even though the Arctic atmosphere is entirely processed by heterogeneous reactions on the surface of ice and water droplets that convert inert chlorine molecules to ClO.

Yet from initial amounts of the order of 450 Dobson units (DU), Arctic ozone concentrations have been falling to about 300 DU in the spring. (Baseline ozone amounts in the Antarctic before the emergence of the 'ozone hole' were 300 DU; they now routinely fall to about 100 DU.) So why are we now seeing these large ozone losses in the Arctic? The answer appears to be longer stratospheric winters. Even though there is little denitrification, temperatures do remain cold long enough to tie up nitrogen as HNO₃ well into the sunlit period in late winter and early spring, and to cause sporadic reprocessing of the air by heterogeneous reactions in polar stratospheric clouds. The temporary lack of nitrogen allows more ozone removal; and, because absorption by ozone warms the

stratosphere, these losses feed back and may keep the stratosphere cold later into the spring. In fact, Rex *et al.*² saw a region in the Arctic in early 1996 where some denitrification occurred and ozone loss continued unabated for more than two months.

It is easy to describe the processes leading to large ozone losses in the Arctic in recent years, but much harder to understand the quantitative details. Unusually large downward trends in ozone concentration have been observed in northern midlatitudes over the past decade and a half; but we still don't know how much of that is due to Arctic polar processes, and how much is a result of midlatitude chemistry or dynamical changes. Also, many details of the heterogeneous chemical mechanism are not established. For example, what form do the ice particles take at temperatures of 190–200 K? Nor do we understand to what extent variability in dynamics, temperature and aerosol concentration determine the year-to-year variations in Arctic ozone.

Chlorine concentrations in the troposphere have peaked and are beginning their expected slow recovery, as the Montreal Protocol, limiting the use of chlorofluorocarbons (CFCs), begins to take effect. Stratospheric chlorine concentrations should begin to decrease soon, and ozone should then begin recovering to earlier amounts. Working out the details of Arctic ozone loss is imperative if we want to recognize ozone recovery at the earliest possible date.

How do we expect this recovery to pro-

Fluid dynamics

How coffee leaves its mark

"Compared with the giants of quantum physics, we soft-matter theorists look like the dwarfs of German folk tales," says Pierre Gilles de Gennes. "We are strongly motivated by industrial purposes. We see fundamental problems emerging from practical questions." Such humility is taken to new extremes by Sidney Nagel and co-workers (Deegan, R. D. *et al.* *Nature* 389, 827–829; 1997), who find insight into the physics of soft matter by means of an eminently familiar, even mundane, phenomenon: the coffee stain.

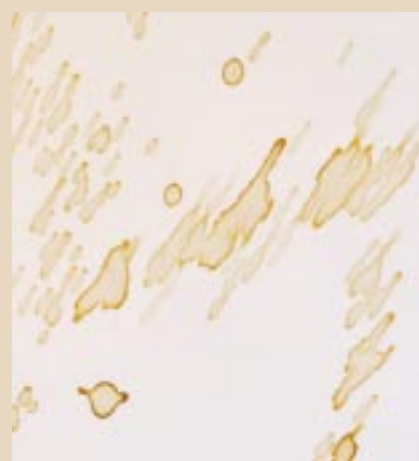
Readers could be forgiven for assuming that the physics of an evaporating coffee droplet would be buried in the annals of a previous century. But how liquid droplets flow, spread and dissipate is imperfectly understood. The statics of the situation are explained by Thomas Young's famous condition for wetting (1805), based on the balance of surface tensions at the droplet edge. But what of the dynamics?

The dynamical situation is often complicated by the fact that the edge of the droplet (the contact line) gets 'pinned' at points on the surface, generally as a result

of surface heterogeneities. This is what underlies the work of Nagel *et al.*, who propose an explanation for why coffee stains have pronounced boundaries, where most of the material is deposited. This material is uniformly distributed in the initial drop, so why does it get concentrated around the edges?

If the edge of the evaporating droplet is pinned in place, evaporation cannot simply shrink the droplet's lateral dimensions. Instead, there must be a net flow towards the edge to replenish liquid lost by evaporation while keeping the contact line in place. Suspended material is carried along with this flow, as shown by video microscopy of polymer microspheres in a drying droplet.

The idea can be made quantitative by calculating the evaporative flux from the surface of a circular droplet as a function of radial distance. It turns out that, in a dilute solution, the flux and flow velocity become infinite at the contact line. That implies complete transfer of the solute to the perimeter by the time the droplet dries out, leaving a precisely sharp ring stain. In



practice, this is modified in a concentrated solute, which leaves a finite ring width and thus the sort of graduated stains seen here.

Industrial processes that might benefit from these ideas are the drying of ink droplets in printing and of surface coatings applied as liquids; and perhaps the efforts of amateur water-colourists, to which de Gennes has also likened soft-matter theorists: "spending their Sunday afternoons in the park, and capturing a few simple scenes."

Philip Ball

R. D. DEEGAN *ET AL.*

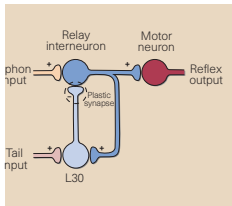


Figure 1 A cloud in the Arctic stratosphere. Ozone losses occur in the air that has been chemically processed by such clouds.

ceed in the Arctic? The answer depends on what we expect to happen to the temperature, which is closely related to why the past few stratospheric winters have been so cold. There are three broad possibilities:

(1) The temperature was low and the winter longer mostly because there was less ozone to absorb solar radiation. (That is, an initial effect was amplified by the feedback mentioned above.) This would imply that as ozone recovers, the temperature will recover with it. The timescale for ozone recovery would be similar to that expected in 'normal' winters.

(2) The temperature was low as part of a

climatological trend independent of ozone — perhaps via CO₂ molecules radiating atmospheric heat to space. This would imply that ozone will not recover rapidly, and may remain low for another decade or more. It may be that ozone concentrations after a cold winter with 2 p.p.b.v. of chlorine are as low as those for a warm winter with 3 p.p.b.v. of chlorine.

(3) The temperature was low because of climatological variability unrelated to ozone or a climate trend. This would imply that ozone recovery will occur on the timescale of 'normal' winters, but with considerable year-to-year variability which will make it difficult to deduce when the recovery begins.

The answer is probably some combination of the above. A great improvement in our knowledge of past interannual variability and trends is necessary if we are to predict the recovery of Arctic ozone as man-made, ozone-destroying chemicals decline, and understand how other sources of stratospheric pollution, such as supersonic aircraft, may influence Arctic ozone. □

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Developmental neurobiology

Neurotrophins moving forward

John V. Heymach Jr and Barbara A. Barres

The changing size, form and connectivity of our brains — during both development and evolution — are thought to depend on trophic feedback between neurons and the target cells that they innervate¹. An attractively simple model for how this trophic feedback works is the neurotrophic hypothesis². The idea is that the number of neurons is matched to the number of target cells that they innervate by a competition for limiting amounts of target-derived peptide factors. These factors, called neurotrophins, promote survival of the presynaptic neurons by retrograde (target to neuron) signalling (Fig. 1). Thus, it now comes as a surprise to learn, in the report by Altar *et al.*³ on page 856 of this issue, that the same neurotrophins can also act by anterograde (neuron to target) signalling.

The neurotrophins are a family of homologous proteins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). The importance of retrograde signalling by neurotrophins during normal development is well established. For instance, when axons are severed or target cells ablated, the presynaptic cells generally atrophy and die. But these consequences can be prevented if exogenous neurotrophins are applied.

What is less well recognized is that, when axons are severed, anterograde degeneration also occurs — postsynaptic cells lose spines and dendrites, atrophy and even die. For example, the survival of some neurons in the chick optic tectum, a visual centre in the midbrain, depends on trophic signals that are supplied by their presynaptic (afferent) inputs from retinal ganglion cells⁴. Neurotrophins are attractive candidates for these

signals because their pattern of immunoreactivity in axon processes indicates that they may be transported anterogradely^{5–7}. Moreover, exogenous BDNF can prevent the death of neurons that have had their afferents removed⁸. And when NT-3 is injected into the chick eye, it is taken up by retinal ganglion cells, transported anterogradely, and released in the optic tectum where it supports the survival of tectal neurons⁹. But these experiments have fallen short of answering whether endogenous neurotrophins are normally involved in anterograde signalling.

The study by Altar *et al.*³ now provides direct proof that endogenous BDNF is anterogradely transported, and that the amounts transported are large enough to alter the phenotype of target cells. Using a highly specific antiserum to detect BDNF protein, they showed that it is widely distributed in nerve terminals. Remarkably, BDNF is even found in regions of the brain that lack BDNF messenger RNA, such as the striatum. Because the presynaptic cells that innervate the striatum produce both BDNF mRNA and protein, these results strongly suggested that the cortical cells anterogradely transport BDNF to the striatum.

To test this possibility directly, colchicine (a drug that disrupts microtubules) was used to inhibit axonal transport. The immunoreactivity of BDNF markedly increased in the cell bodies of the cortical neurons, and it decreased in the striatum. Furthermore, cortical ablation — which eliminates the striatal afferents — eliminated the axonal BDNF immunoreactivity in the striatum, and decreased the amount of BDNF there by 66 per cent. (Much of the residual BDNF was shown to derive from other afferent pathways.) In contrast, ablation of the striatal neurons with an excitotoxin did not decrease the levels of BDNF in the striatum. So, the bulk of striatal BDNF must be anterogradely transported from the cortex. Because almost all of the BDNF in the striatum is present in the terminals of afferent neurons, and

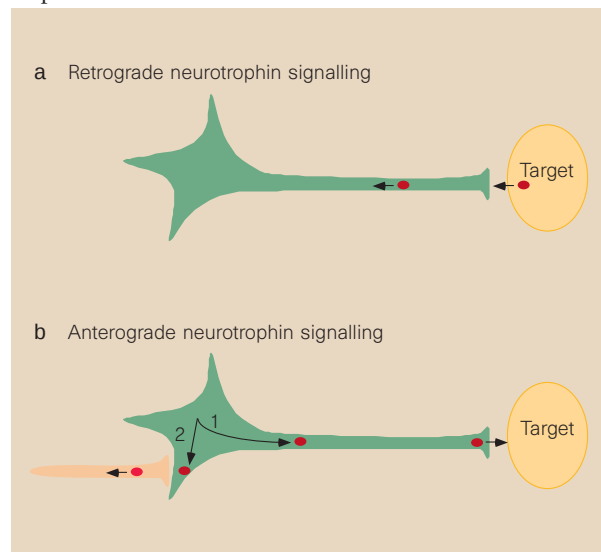


Figure 1 Retrograde and anterograde neurotrophin signalling. a, Target-derived neurotrophin (red circle) is taken up by an innervating (afferent) neuron, and retrogradely transported back to the cell body, where it affects survival and differentiation. The target may be neuronal or non-neuronal. b, The neuron may direct its neurotrophin down the axon for anterograde, neuron-to-target signalling as shown by Altar *et al.*³ (1), or to its dendrites or soma (2), for uptake and retrograde transport by an afferent (presynaptic) neuron.

the number of striatal neurons that express the phenotypic marker parvalbumin is decreased in transgenic mice that lack BDNF, the findings of Altar *et al.* indicate that release of anterogradely transported endogenous BDNF is likely to be functionally significant.

Does such anterograde neurotrophin signalling occur elsewhere? BDNF protein and mRNA are distributed throughout the nervous system, suggesting that both retrograde and anterograde^{5–7} signalling are probably widespread. So a neuron could, perhaps, 'choose' to engage in either anterograde or retrograde signalling by directing its neurotrophin to a different location (Fig. 1). In this case, what determines the destination of a neurotrophin once it is produced by a neuron? Neurons have a variety of mechanisms for targeting proteins to different cellular locations. One possibility is that the direction of neurotrophin sorting is determined by the developmental stage of a neuron so that, for example, only mature neurons have the cellular machinery in place to ship neurotrophins anterogradely. Another possibility is that members of the neurotrophin family are targeted differently, although no striking differences have been detected so far^{10–12}. Alternative splicing, mRNA targeting or post-translational modifications of neurotrophin precursors may also be involved. Or neurons may express carrier proteins which direct a given neurotrophin to a specific destination.

What would be the point of anterograde BDNF release? It could be involved in regulation — stimulating the growth of spines and dendrites, and other aspects of neuronal phenotype, for instance¹³. Or, perhaps BDNF acts as a peptide transmitter and modulator of synapses, given that it potentiates synaptic transmission¹⁴, that it is released in an activity-dependent manner^{12,13}, and that its receptor is clustered postsynaptically¹⁵. It is already clear that there is a dynamic interplay between electrical activity and neurotrophins. Electrical activity stimulates the postsynaptic production and release of neurotrophins¹³, and it also increases the responsiveness of neurons to neurotrophins¹⁶. So anterogradely released BDNF could have both short- and long-term effects on the formation and function of neural connections.

In any case, although the neurotrophic hypothesis has provided a useful framework for considering neurotrophin signalling, Altar and colleagues' results indicate that the reality is more complex. Rather than a passive, retrograde, trophic-feedback system, dynamic pre- and postsynaptic interactions between activity and neurotrophins are, undoubtedly, crucial in sculpting the developing brain into its final, functional form. □

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Synapses

Plastic plasticity

John H. Byrne

Over the past 20 years neuroscientists have become increasingly struck by the enormous capacity of the nervous system for adaptation. The latest development appears on page 860 of this issue¹, where Fischer and colleagues describe analysis of a deceptively simple neural system in the mollusc *Aplysia*.

One of the main sites that expresses adaptation in the nervous system is the synapse — a structure, unique to neurons, where chemical messengers are released by nerve impulses in the presynaptic terminal and diffuse across a small distance to produce electrical changes in the target, postsynaptic, neuron. These sites of interneuronal communication are far from the rigid 'solder joints' they were once thought to be. Rather, nerve impulses alter the process of synaptic transmission so that the ability of subsequent impulses in the presynaptic neuron to influence the postsynaptic neuron

is enhanced (or in some cases reduced).

This activity-dependent synaptic plasticity provides dynamic control of the flow of information in neural networks; it also serves as a 'memory' that the circuit had been active. Recently, we have also begun to learn that synaptic plasticity itself is regulated — a phenomenon that has been called metaplasticity (see ref. 2 for review). Several examples have been reported^{3–5}, but virtually nothing is known about mechanisms of metaplasticity or its behavioural consequences.

Fischer *et al.*¹ now take the analysis of metaplasticity several steps forward. They show that related forms of synaptic plasticity, known collectively as augmentation and post-tetanic potentiation (augmentation/PTP), are suppressed by the activation of a second

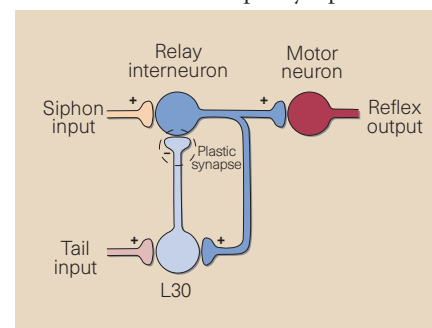


Figure 1 Simplified version of a neural circuit that controls a defensive reflex in the mollusc *Aplysia*. Touching the siphon skin activates (+) a relay interneuron. The interneuron activates a motor neuron that elicits the reflex. A negative-feedback circuit is provided by interneuron L30. The inhibitory connection (–) from L30 increases in strength when L30 is active, which decreases the ability of siphon stimulation to elicit the reflex. If the tail is touched before the siphon, the braking effects of the L30 pathway are more profound because the tail-induced L30 activity has strengthened the plastic synapse. A third pathway, shock — and the administration of the neurotransmitter serotonin (5-HT), not shown — modulates the plasticity of the plastic synapse so that the braking effects are reduced.

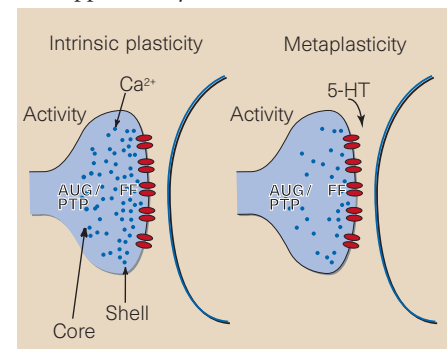


Figure 2 Possible mechanisms for metaplasticity. Calcium influx through calcium channels (red) in the presynaptic terminal leads to an influx of Ca^{2+} during activation which triggers two forms of synaptic plasticity — frequency facilitation (FF) and augmentation/PTP (AUG/PTP) — that are intrinsic to the L30 synapse. The molecular processes underlying these forms of plasticity are assumed to reside in different places in the presynaptic terminal: frequency facilitation at the 'shell'; and augmentation/PTP at the 'core'. Normally, enough Ca^{2+} enters during L30 activation to activate both processes. After shock to the animal (and application of 5-HT), less Ca^{2+} is assumed to enter. The reduced Ca^{2+} in the shell is still sufficient to activate frequency facilitation; but less Ca^{2+} diffuses to the core and so there are lower levels of augmentation/PTP — and thus metaplasticity.

modulatory pathway, and they provide insights into the underlying mechanisms. One particularly striking aspect of this study is the authors' ability to relate the metaplasticity to the behaviour of an animal. Fischer *et al.* take advantage of a well-described circuit that mediates the defensive siphon-withdrawal reflex in the mollusc *Aplysia*. At its elementary level the circuit consists of the serial cascade of three types of neurons — sensory neurons that propagate the sensory input to the central nervous system; motor neurons that cause the siphon to withdraw; and excitatory interneurons that relay the information from the sensory neurons to the motor neurons (see Fig. 1; the sensory neurons also connect directly to the motor neurons but this connection has been omitted for clarity).

The understanding of the operation of the circuit would be rather trivial if it were not for several additional features. First, the circuit contains a negative feedback loop in which the relay interneuron excites another interneuron (cell L30), whose activation leads to inhibition of the relay interneuron. Second, L30 can be activated by another sensory pathway from the animal's tail. Third, the inhibitory synapse between L30 and the interneuron exhibits activity-dependent synaptic plasticity. Fourth, the plasticity of the L30 synapse is itself regulated.

The L30 interneuron exhibits two related, but mechanistically distinct, forms of activity-dependent plasticity that are intrinsic to its synapse. During a burst of nerve impulses, the strength of the synaptic connection steadily increases, a phenomenon known as frequency facilitation. But for a period of about one minute after the burst (tetanus), impulses in L30 continue to produce larger responses — this is the augmentation/PTP phenomenon. The negative feedback loop acts as a built-in braking mechanism to limit activation of the motor neuron by sensory input. The brakes can be applied to an even greater extent if a stimulus to the tail is delivered first, this stimulus activating L30 and inducing augmentation/PTP at its synapse. Now, if the siphon is stimulated during a time when the 'memory' for skin stimulation represented by augmentation/PTP is still present, the feedback inhibition will be even greater than before. So this form of synaptic plasticity has a powerful effect on regulating the gain of the reflex circuit.

Fischer *et al.* find that another input pathway, shock to the animal, suppresses the synaptic plasticity (that is, augmentation/PTP) and thus reduces the effectiveness of the feedback inhibitory circuit. Interestingly, shock affects one form of plasticity (augmentation/PTP) but not the other (frequency facilitation), indicating that the modulation of the plasticity is specific to only certain forms of plasticity at this synapse.

Finally, the authors have begun to examine the mechanisms of the metaplasticity.

They found that the neurotransmitter serotonin (5-HT), an agent implicated in modulating the properties of many neurons in *Aplysia* and other animals, mimicked the modulatory effects of shock in that it reduced augmentation/PTP but not frequency facilitation. Similar effects were obtained by reducing the level of extracellular calcium, an ion known to be important for inducing both forms of plasticity. The implication that still needs to be examined directly is that the metaplasticity involves a reduction by 5-HT of calcium influx, or intracellular levels of calcium, so that augmentation/PTP is modulated. The specificity of the metaplasticity to augmentation/PTP, but not frequency facilitation, could be explained by a model in which the two types of plasticity are induced at different sites within the presynaptic terminal

(Fig. 2; see refs 6 and 7 for review).

How general are the results? It is too early to say, but the phenomenon of augmentation/PTP is ubiquitous and it seems likely that its type of regulation would be important in many neural systems. □

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Earth science

Probe of a plate interior

Bruce Yardley

The German Continental Deep Drilling Program (KTB) has been a remarkable attempt to learn more about one of the least understood parts of the outer Earth — its crystalline continental crust. But reports from the project have not circulated widely, hence the value of a special set of papers in *Journal of Geophysical Research* (102, No. B8; 1997) which is intended to bring the main scientific work of the KTB project to a wider audience.

In drilling through the top third of the crust, the project has provided a laboratory to relate deep-rock properties to those inferred from surface measurement, and has also shown that the best predictions of what rocks would be encountered at depths of just a few kilometres were woefully inadequate. Drilling lasted from September 1987 to October 1994, during which time a 4-km pilot hole was drilled and cored, followed by a 9.1-km main hole. Extensive downhole logging and continuing experimentation has produced a vast amount of data.

The results of the project fall into two categories: geological data that bear on the development of the Variscan orogen in central Europe (the roots of a mountain chain formed in Carboniferous times), and geophysical information about the state of the Earth's crust today in a reasonably stable plate interior. By far the most important regional result is that the geology (and also the heat flow) encountered by drilling was very different from that predicted by a four-year programme of site investigation. Instead of passing rapidly through a thin sheet of crystalline rocks into a diverse stack of tectonic units beneath, the drillers encountered over 9 km of uniform high-grade metamorphic rocks, which did not

even vary significantly in their conditions of peak metamorphism (see Fig. 1).

Geologists and geophysicists working in crystalline rocks have known for years that there are real difficulties in interpreting two-dimensional seismic-reflection profiles in parts of the crust where many major features are steep, whereas properties that give rise to shallow reflectors are not necessarily of fundamental importance. Never before has anyone spent DM528 million (US\$303 million) proving it, however. As drilling continued, a three-dimensional seismic survey was shot and the processing developed to the point where it did become possible to predict the presence of steep-dipping features deep in the hole (H.-P. Harjes *et al.*, pp. 18,267–18,288).

To judge the success or otherwise of the KTB drilling on the extent to which it vindicated the geological prognosis for the site would be to completely miss the point of the project, however. To have found what was predicted would have been a real waste of money. The 19 papers in this set deal with aspects of the nature of the continental crust which could not have been investigated without deep drilling, and they answer some questions that have beset crustal geology. The main value of the hole lies in the downhole experimentation and in the temperature reached at the bottom of the hole, about 265 °C, rather than in the depth itself. This temperature approaches the onset of the brittle–ductile transition in the crust, on which many rock properties depend, and unfortunately it presents a major obstacle to further drilling at high confining pressures, because of the distortion of the drill hole itself. The Russian deep hole in the Kola peninsula, drilled in the 1970s and 1980s, was deeper, but

Daedalus

Floating on nothing

The lightest possible filling for a balloon is not hydrogen, but vacuum. But how to make it strong enough to withstand the pressure of the atmosphere, while still being light enough to rise? Daedalus's answer is a graded-cell polymeric foam.

Inspired by that foaming urethane monomer, he is devising a new liquid monomer that polymerizes with release, not of carbon dioxide, but hydrogen. It will be placed in a hollow, evacuated, spherical mould which will be spun rapidly about three axes simultaneously. The resulting uniform, radial, centrifugal force field will spread the monomer over its interior surface as an even coating. When it begins to foam, the bubbles will swell towards the centre. Their strong expansion into the central vacuum will be opposed by the centrifugal field. But this field, of course, declines towards the centre of the spinning sphere. So the more the foam advances towards the centre, the more its cells will expand, and the lower the gas pressure within them. When the reaction is over, the mould will contain a spherical foamed balloon. Its smooth outer skin will be supported by a foam whose cells grow ever larger, and contain an ever lower pressure of hydrogen, as they extend towards the centre. The largest cell, the remains of the original interior space, will occupy the central region, and will contain vacuum. At each radius, the cellular foam will just be strong enough to support the difference of pressure across it. Thus the balloon as a whole will safely withstand atmospheric pressure.

Released into the atmosphere, the new rigid vacuum balloon will rise until it reaches that height at which it has the same density as the ambient air. There it will float indefinitely, drifting randomly around the globe with the winds. The obvious use is in cellular telephony. In this application, each balloon will carry a solar-powered radio repeater, and will be designed to float perhaps 20 km up, safely above the commercial air lanes. For this height, about a thousand balloons wandering at random should ensure that anywhere on Earth, at least one will be above the horizon. They will maintain effortless global coverage far more cheaply than any satellite system. Like satellites, they will have a limited life, but will be much easier to replace. As fast as air diffuses into them, or their electronics fails, they will be punctured by laser beams fired from aircraft, and new ones lofted up to replace them.

David Jones

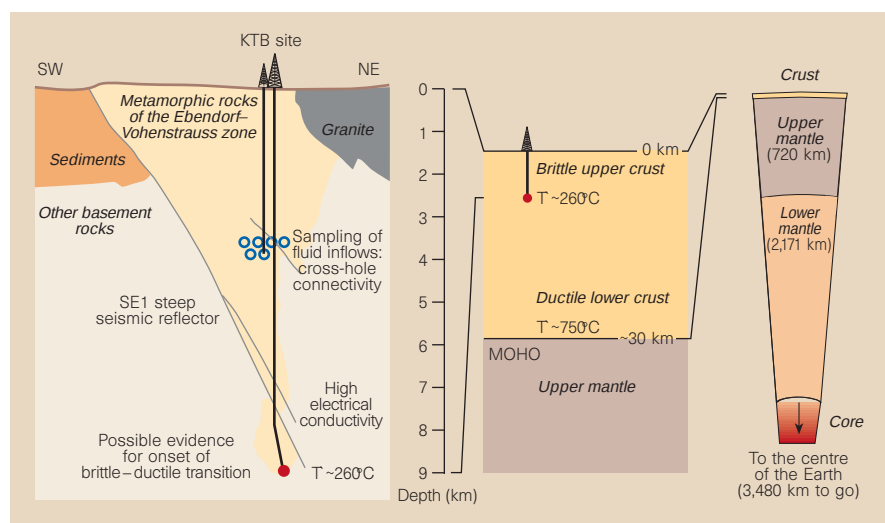


Figure 1 The KTB drilling project, in context. a, This current schematic view of the deep geology of the KTB site, summarized mainly from R. Emmertman and J. Lauterjung (*J. Geophys. Res.* 102, 18,179–18,202; 1997), shows how the deep drill hole passed fortuitously through 9 km of rather uniform high-grade metamorphic rocks, rather than the predicted stack of tectonic slices. However, insights were obtained on chemical and physical properties of the crust at depth, and some of these are also indicated. Parts b and c put this direct observation in the context of the continental crust as a whole and of the rest of the Earth.

less thoroughly monitored, sampled and investigated.

One of the highlights of the KTB project has been the information it has provided about deep crustal fluids, summarized by P. Möller *et al.* (pp. 18,233–18,254). Not only has the presence of highly saline, near-stagnant NaCl–CaCl₂ brines been confirmed, but samples have been collected and analysed (notably at 4,000 m) and the permeability structure of the rocks investigated. The brines are at near-hydrostatic equilibrium and occur in interconnected fractures surrounding blocks of very low-permeability crystalline rock, which retain primary isotopic characteristics — demonstrating that they have not experienced pervasive fluid flow either during metamorphism or subsequently.

Fluid flow also plays a role in explaining one of the surprising results of the drilling, the heat flow at depth. Based on near-surface measurements, it was expected that the heat-flow density would be about 55 mW m⁻², with a temperature gradient of 21 °C km⁻¹. Below 1,600 m, however, both the pilot and main holes showed a marked increase in heat flow and temperature gradient to 85 mW m⁻² and 28 °C km⁻¹, respectively. C. Clauser *et al.* (pp. 18,417–18,442) ascribe this to the combined effects of advection of groundwater and a memory of the lower surface temperature during the Pleistocene, more than 10,000 years ago.

Electrical conductivity of the continental crust has been a particularly contentious issue, especially the question of whether it is a guide to the water content of the lower crust. The KTB borehole passed into a zone of anomalous conductivity more or less as predicted, which is itself a significant vindication

of field conductivity measurements, and the ELEKTb collaboration, an interdisciplinary group from several German institutions (pp. 18,289–18,306), conclude that it is caused by the presence of continuous graphite films within anastomosing fault zones. This result contradicts both of the most popular current hypotheses: abundant fluids or pervasive graphite films through the grain boundary network of the rock matrix.

Several studies have indicated that the state of stress in the upper crust is close to frictional equilibrium on pre-existing faults. Measurements in the KTB hole allowed M. Brudy *et al.* (pp. 18,453–18,476) to calculate the complete stress tensor and confirm that this pattern is maintained to depths of at least 8 km. Transmission electron microscope studies of quartz retrieved from the final depth suggest that, there, recovery has begun to take place, and the stress may be less than that indicated by extrapolation from higher levels. G. Dresen *et al.* (pp. 18,443–18,452) draw the controversial conclusion that the hole reached the onset of the brittle–ductile transition. This implies the effective decoupling of middle and upper crust, and should force geologists and geophysicists to confront the very different views that they hold about processes in stable continental regions.

The KTB project has been one of the major Earth science experiments of the twentieth century, transforming our understanding of the upper continental crust. It is good to see so many of the results gathered together and publicly available — this issue of *Journal of Geophysical Research* is essential reading for all Earth scientists. □

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Obituary

Hans Jurgen Eysenck (1916–97)

Psychologist who created the modern scientific theory of personality

Mention 'science of personality' and you may be accused of oxymoron. Yet this science exists, is important and flourishes. It is largely the creation of one man — Hans Eysenck — who died on 4 September, aged 81.

Eysenck was born in Berlin, but left in 1934 because he abhorred Nazism. He came to London University with the intention of reading physics, but his previous studies failed to meet the admission criteria. So he registered for psychology as the nearest thing to science that they would allow.

When Eysenck began his work in the 1940s the field was, frankly, a mess. Thousands of personality traits had been identified, labelled and measured, mainly by short questionnaires. Attempts to simplify this profusion were based on multivariate statistical analyses. These aimed to reduce the surface pattern of correlations between all possible pairs of measurements to a smaller number of underlying factors which could then, statistically speaking, reproduce the main features of the surface pattern. But there was no agreement on the correct form of analysis, although everyone except Eysenck agreed that a purely statistical solution would be forthcoming (if only it were possible to persuade the others that one's own was the best!).

Eysenck cut through the verbiage with a first, cardinal insight — no solution can be established on purely statistical criteria, and any suggested solution can serve only as a hypothesis which then needs to be put to the standard scientific tests of prediction, test and verification. He showed that all of the solutions on offer were mathematically equivalent and intertranslatable. So the argument was not about the maths, but about which version of the maths best applied to the true 'structure of personality'.

If there is such a thing as a structure to personality, what gives rise to it? Eysenck's second great insight was that the source could lie only in the organization of the brain: whatever genetic, environmental or social influences contribute to a person's individuality, they must have acted by shaping the way in which the brain functions. However, certainly in the 1940s and 1950s, it was impossible to study the



relevant features of brain function directly, so inferences needed to be made from experimental studies of behaviour. In this strategy, as he acknowledged, Eysenck followed in the footsteps of Ivan Pavlov. But whereas Pavlov started from observations of behaviour in animals and speculated about human personality, Eysenck started with the measurement of individual differences in human behaviour and sought explanations in the mammalian brain on the basis of animal behaviour. Because he had to draw on what was known in these more basic, relatively underdeveloped fields, the details of his extraordinarily all-encompassing theory of personality were bound to be, in many respects, wrong. Yet he sketched the basic logical shape that such a theory should take in a way that, I believe, will be lasting.

At the time, almost everything that Eysenck wrote on the topic was the subject of hot dispute. Today, there is widespread agreement that his essential postulates were correct. For example, in the best traditions of scientific parsimony, he held that there are only a few (three, in his view) fundamental and independent dimensions that define the space in which human personality can vary. He doggedly defended this position against an opposition that has retreated from an initial minimum of 16 to a modal five at present, two of which (extraversion and neuroticism) are identical to his. He also held that much of the variance along his

three main dimensions of personality

(psychoticism being the third) reflects the cumulative action of additive polygenes, and that psychiatric disorder is often due to extreme positions on the resulting continuous distributions of vulnerability. This view — which was initially rejected out of hand by clinicians and social scientists alike — is now so generally accepted that it has sparked an international race to identify the genes that determine high levels of neuroticism and, thus, disorders of anxiety and depression.

Undoubtedly, the construction of a scientific theory of personality was Eysenck's most important and enduring achievement. For this alone he deserved the honours which, in his own country, he (scandalously) never received. In part, the reason for this neglect lies in his activities as advocate and polemicist. His first battle was against psychoanalysis. In 1946, Eysenck was appointed to the Institute of Psychiatry (where he spent the rest of his career) by the psychiatrist Aubrey Lewis. One of his objectives was to start up a training course for clinical psychologists. Having scoured the United States (which was then dominated by psychoanalysis), he spurned all of the examples that he found there and established his own new training model, based on evidence from basic and clinical experimental research.

This model has since been copied throughout the world, and it has contributed much to what has become the most effective method of treating a range of neurotic disorders — cognitive-behavioural therapy. Although Eysenck made little direct contribution to these therapeutic advances, his advocacy was very important in spreading the word. But in the process he alienated a whole generation of psychodynamic psychiatrists. To these he soon added other enemies, as he espoused a variety of politically incorrect causes: he argued that Communists and Fascists have similar personalities; that one cannot exclude the possibility that race- or sex-differences in IQ are, at least in part, genetically based; and that the correlation between smoking and disease may be mediated by a common factor, such as personality type.

At a personal level, Eysenck was the kindest and most courteous of men. In print, however, he loved a fight, using weapons of the intellect but with no holds barred. For this pugnacity his reputation undoubtedly suffered; but his position as the most highly cited psychologist of his generation will, I believe, be matched in the history books.

Jeffrey Gray

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Prion research: the next frontiers

Adriano Aguzzi and Charles Weissmann

Although the precise structure of the infectious agent that causes prion diseases is still unknown, important features of its molecular genetics have been elucidated. The transmissibility of BSE to humans dramatically highlights the need to understand prion biology fully.

The study of transmissible subacute spongiform encephalopathies (TSEs) — to which sheep scrapie, bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob disease (CJD) belong — is fraught with difficulties. The causative transmissible agent (the 'prion') has tenaciously eluded all attempts at definitive identification. Moreover, it can be assayed only in experimental animals and, because the delay between inoculation and disease is measured in months, the turnaround time of experiments limits the number of ideas that can be tested.

Most TSE researchers adhere to the 'protein-only' hypothesis (see box, 'A brief history of prions'), which maintains that the transmissible agent is devoid of nucleic acid. But some continue to be convinced that these diseases are caused by a sort of virus. Notwithstanding the difficulties, the field of prion biology has witnessed considerable advances in the past few years. Rather than reviewing these in detail, our main purpose here is to highlight the most pressing, as-yet-unresolved questions, and to offer some thoughts on how these might be addressed.

Will the true Mr Hyde please stand up? Undoubtedly, a high priority is to characterize the infectious agent. An impressive wealth of circumstantial evidence, mainly genetic, supports the protein-only hypothesis. The main incarnation of this theory is the proposal that PrP^C — a normal protein found in the brain and other organs of all vertebrates examined so far — can be converted into a misfolded, pathological form (designated PrP^{*})¹ by some kind of autocatalytic process. In most forms of the disease, this is accompanied by the formation of PrP^{Sc}, an insoluble form of PrP that is largely resistant to enzymatic digestion by proteases. Stanley B. Prusiner and his colleagues propose that PrP^{*} and PrP^{Sc} are the same, and they believe that the protein-only hypothesis best explains the sum of all available evidence — a view now enshrined by the award of the 1997 Nobel Prize in Physiology or Medicine to Prusiner. But the opposing faction is still demanding an irrefutable experiment to establish that prions are solely composed of protein. As

Popper pointed out, however, whereas a theory can be falsified it can never be proved.

The three-dimensional structure of mouse PrP^C has been largely unravelled² (Fig. 1, overleaf). Its carboxy-terminal half consists of three α -helices and a short β -pleated sheet which form a stable globular structure; the amino-terminal half is a random coil. Determining the structure of PrP^C has been difficult, but it will be an even tougher act for PrP^{Sc}. Fourier-transform infrared and circular-dichroism studies have shown that, whereas PrP^C has about 43% α -helix and 3% β -sheet, preparations of PrP^{Sc} have around 34% α -helix and 43% β -sheet, suggesting that the β -pleated domains arise at the expense of the unstruc-

tured amino-terminal region. Moreover, preparations of purified PrP^{Sc} are insoluble, and they are contaminated with other substances including an unknown 'aggregating compound' that is covalently linked to fatty acids³, among other things. If nucleic acids are present at levels that are stoichiometric with infectious units, they must be shorter than 100 nucleotides⁴. Solubilization of PrP^{Sc} preparations is accompanied by denaturation and loss of infectivity, and although protease-resistant insoluble PrP can be recovered through appropriate renaturation, infectivity has so far never been regained.

These technical obstacles have hindered determination of the structural changes that occur during the transition of PrP^C to PrP^{Sc}. Perhaps it will be necessary to resort to unorthodox approaches, such as the introduction and localization of intramolecular crosslinks to study the folding changes. But the main problem is the fact that the ratio of infectious units to protease-resistant molecules is in the order of 1:100,000 or less. Is this because the infectious unit is an aggregate of many identical PrP molecules? Or is the infectious unit a minor — but distinct — component in a mixture of different PrP molecules? It may be worth using one of the models of prion disease in which little or no PrP^{Sc} accumulates, and yet high levels

A brief history of prions

In 1939, Cuille and Chelle discovered that scrapie was transmissible to experimental animals, leading to recognition of the unusual properties of the infectious agent — in particular, the long incubation time and resistance to conventional sterilization procedures. The small target size for ultraviolet irradiation and an action spectrum incompatible with that of a nucleic acid led Tikva Alper to suggest that the infectious agent might be a protein. Shortly afterwards, J. S. Griffith outlined the 'protein-only' hypothesis, which states that the infectious agent consists of the modified form of a normal cellular protein.

In 1982, Stanley B. Prusiner achieved biochemical purification

of the scrapie agent²⁹ which he called 'prion', and equated it with 'prion protein' (PrP), the major component and principal protein identified in the infectious preparation. Based on partial sequences of PrP, the cognate complementary DNA and gene were cloned and, to everybody's surprise, they turned out to be encoded by a human gene, *Prnp*^{30,31}. This led to the recognition of two forms of PrP — the normal form, PrP^C, and a pathological form present only in scrapie-infected organisms, PrP^{Sc}. These findings (which were eventually extended to human TSEs) provided an experimental basis for the protein-only hypothesis.

The primary amino-acid sequence of PrP was identified as a main determinant of the species barrier of prions⁸. Linkage was demonstrated between familial forms of prion diseases and mutations in the prion gene³². PrP-knockout mice were found to be resistant to infection with prions³³ and, finally, it was shown that knockout mice containing transgenic PrP with certain mutations succumbed to neurological, scrapie-like disease, generating infectious agent in their brains³⁴. Although a nucleic-acid-containing infectious agent, or 'unconventional virus', has also been invoked as causative agent, we have no data to indicate a scrapie-specific nucleic acid.

A. A. & C. W.

of infectivity are produced, to find a more tractable system.

Wanted: the philosopher's stone

The current holy grail of the protein-only faction is to produce prions *in vitro* from components that have never been in contact with the infectious agent — that is, from PrP made in healthy eukaryotic cell lines, *Escherichia coli* or, better still, chemically from amino acids. Yet, so far, it has not even been possible to denature prion preparations and renature them to regenerate measurable levels of infectivity. Hopes have been raised by *in vitro* conversion of PrP^C into a protease-resistant isoform by incubation with PrP^{Sc}, particularly because the results reflect some of the biological properties of the transmissible agent, such as the species barrier and strain specificity⁵. But all that glitters is not gold, and protease resistance of PrP should not be equated, *a priori*, with infectivity. Not only can prion disease appear in the absence of detectable levels of PrP^{Sc} (ref. 6), but protease-resistant PrP with no infectivity can be generated⁷. So there may be several structures of PrP that differ from that of PrP^C, the infectious form of which, PrP*, may or may not coincide with a protease-resistant structure.

The fundamental question — namely, whether the *in vitro* 'conversion' of PrP^C gives rise to infectivity and not only to a protease-resistant isoform — cannot yet be answered. This is mainly because a substantial molar excess of PrP^{Sc} (which is associated with infectivity) over PrP^C must be added to the reaction mixture. Therefore, infectivity generated *de novo* (if any) must be measured against a high background using an imprecise detection assay whereby susceptible mice are challenged with serial dilutions of the agent, and the smallest amount that causes disease is determined.

To overcome the background problem, one could exploit the species barrier. This is characteristic of many prion diseases, and is mainly determined by small but critical differences in the amino-acid sequence of PrP^C (ref. 8). Usually, mice are not susceptible to hamster prions, so if experimental conditions could be found that allowed hamster PrP^{Sc} to convert mouse PrP^C into PrP^{Sc} *in vitro*, an increase in the infectious titre for mice would indicate *de novo* generation of prions. Better yet would be to find conditions that lead to conversion in the absence of any PrP^{Sc} 'template' — recombinant PrP with one or more of the point mutations that are associated with spontaneous disease could be used as a template. Furthermore, the conversion might be driven by the use of an antibody specific for PrP^{Sc}. Or perhaps an 'imprint' of PrP^{Sc}, prepared by polymerizing monomers in the presence of PrP^{Sc}, could be used to promote the conver-

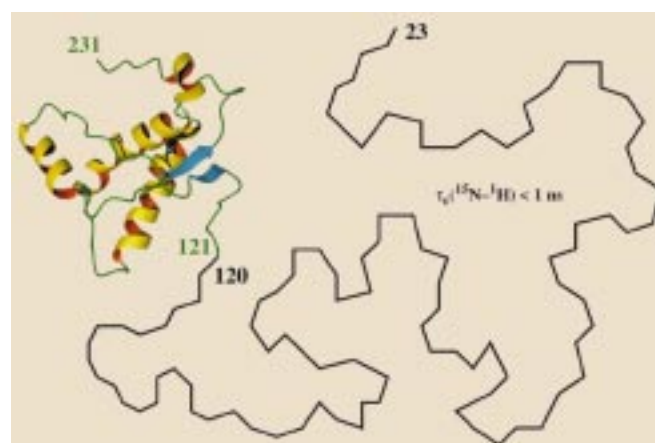


Figure 1 The three-dimensional structure of a recombinant murine PrP^C expressed in *Escherichia coli*. Unexpectedly, the amino-terminal polypeptide segment 23–120 is flexibly disordered. (Reprinted from ref. 2.)

sion⁹. But would a successful conversion experiment dissipate all doubts on the nature of the prion? Perhaps not, because the die-hard pro-virus faction may then argue that the protein is not truly the infectious agent, but that it merely induces a latent, ubiquitous virus.

Straining the imagination

Many distinct strains of scrapie prion have been derived from sheep isolates. These strains have different phenotypes — for example, incubation times, distribution of lesions in the brain, relative abundance of mono-, di- and unglycosylated PrP^{Sc}, and electrophoretic mobility of the protease-resistant part of PrP^{Sc}. But they can all be propagated in the same inbred mouse strain, indicating that, within the framework of the protein-only hypothesis, the same polypeptide chain can mediate different strain phenotypes. The conformational hypothesis postulates that each strain is associated with a different conformation of PrP^{Sc} (or PrP*); see box, 'Definitions'), and that each of these can convert its host's PrP^C into a likeness of itself. This view is supported by the strain-specific differences in electrophoretic mobility of protease-treated PrP^{Sc}, which are due to different cleavage sites for the protease¹⁰.

A further fascinating question regards the mechanism that allows different strains of scrapie prions to target different regions of the brain¹¹. The so-called 'type 4' pattern of PrP^{Sc} — which is typical for BSE and new variant Creutzfeldt–Jakob disease (vCJD)¹² — shows a high ratio of diglycosylated to monoglycosylated molecules, in contrast to types 1–3 (ref. 13). We have interpreted this to mean that mono- and diglycosylated PrP^C show different susceptibilities to different strains¹⁴. Extending this argument, other, more subtle, differences in glycosylation could lead a strain to prefer cells in which that particular glycoform is more heavily expressed. Indeed, over 400 different forms of the PrP molecule may exist due to the diversity of the glycan (sugar) moieties. The diversity of prion strains may come about by a combination of differences in tertiary

structure and glycosylation pattern, perhaps in an interdependent way.

In pursuit of function

The physiological function of the normal prion protein has so far resisted elucidation. Mice devoid of PrP^C develop normally and suffer from surprisingly few defects. A synaptic phenomenon called long-term potentiation (which is presumed to be important for short-term memory and learning) is impaired¹⁵, but no alterations of these behavioural parameters have been detected¹⁶. These mice also have aberrant sleep patterns¹⁷, and one line of PrP-deficient mice has shown degeneration of cerebellar neurons in old age¹⁸. But a causal relationship to the absence of PrP^C remains to be established.

So why has a gene that is not essential been conserved during evolution? Perhaps the function cannot be perceived under laboratory conditions, yet confers selective advantage in the wild. Or maybe PrP^C can be functionally replaced by other, possibly structurally quite different, proteins; conceivably, these might be upregulated during embryonal development in the absence of PrP. They should be searched for by comparing messenger RNAs and/or proteins in wild-type and PrP-knockout mice.

By virtue of its location at the outer surface of cells, anchored by phosphatidylinositol glycolipid, PrP is a candidate for a signalling or (less likely) transport function. Although several candidate ligands have been reported, no interactions have been confirmed *in vivo*. On the basis of genetic experiments, Prusiner and colleagues have postulated that a 'protein X' might interact with PrP^C and be important in its conversion to PrP^{Sc} (ref. 19). Identification of this protein and determination of its function (possibly as a chaperone, to help mediate protein folding) is of the highest interest.

One intriguing thought is that the function of PrP^C may be related to its ability to assume various conformations, by virtue of its long, unstructured, flexible tail (Fig. 1); perhaps the price that has to be paid for this

flexibility is susceptibility to the rare conversion into a pathogenic form. A PrP^{Sc}-related isoform may exist in equilibrium with PrP^C, and the 'seeding hypothesis' (Fig. 2, overleaf) postulates that, when the concentration of such an intermediate exceeds a critical concentration, a stable aggregate of PrP^{Sc} may form and rapidly accrete further monomers.

Although these characteristics may lead to fatal consequences in TSEs, they might be beneficial in a different context. For example, in the case of the {psi+} state in the yeast *Saccharomyces cerevisiae*, the polypeptide-chain release-factor (eRF3, also designated sup35p) can be recruited into a non-functional aggregate by a 'prion-like' process. This allows suppression of otherwise lethal nonsense mutations. Also in *S. cerevisiae*, the Ure2p protein can exist in an inactive, aggregated state that spreads similarly, but can confer a selective advantage under special circumstances²⁰. So perhaps the 'prion principle' may be more widespread than is currently recognized.

Prions and brain damage

It is sobering to realize that very little is understood about the mechanisms by which prions elicit brain damage. They cause vacuolation and death of nerve cells, activation of astrocytes and microglial cells, and the invariably lethal impairment of electrical functions of the brain. The first priority will be to determine whether the damage is caused by accumulation of the pathological prion protein, or by depletion of its normal isoform. Although PrP-deficient mice are healthy¹⁶, this may be because they have had time to adapt to PrP-less life from early development. The crucial experiment could be 'conditional knockout' of the *Prnp* gene, allowing expression of PrP^C to be abruptly shut off in the adult mouse.

If, on the other hand, we accept that PrP^{Sc} deposition is the cardinal event in pathogenesis, why, in many instances of human and experimental TSE, can extremely little (if any) PrP^{Sc} be detected, even in terminal disease⁶? PrP^{Sc} deposition would have to act from within the cell, because PrP^C-deficient nerve cells are not affected by extracellular PrP^{Sc}, even after long-term exposure²¹.

We also need to know what is the contribution of various types of cell in the central nervous system (CNS) to TSE pathogenesis. The prime target seems to be neurons, yet profound neuronal loss is not always seen in TSEs. Instead, astrocytic activation occurs very early, leading to physiological effects such as impairment of the blood-brain barrier. Because astrocytes can support prion replication²², it will be interesting to work out how this cell type is involved in TSE pathogenesis. Growing evidence also incriminates microglial cells in brain damage, not only in TSEs but in diseases ranging

Definitions

● **Prion:** Agent of transmissible spongiform encephalopathy (TSE), with unconventional properties. The term does not have structural implications other than that a protein is an essential component.

● **'Protein-only' hypothesis:** Maintains that the prion is devoid of informational nucleic acid, and that the essential pathogenic component is protein (or glycoprotein). Genetic evidence indicates that the protein is an abnormal form of PrP (PrP* or, perhaps, PrP^{Sc}). The association with other 'non-informational' molecules (such as lipids or glycosamino glycans) is not excluded.

● **PrP^C or PrP-sen:** The naturally occurring form of the mature *Prnp* gene product. Its presence in a given cell type is necessary, but not sufficient, for replication of the prion.

● **PrP^{Sc} or PrP-res:** An 'abnormal' form of the mature *Prnp* gene product found in tissue of TSE sufferers, defined as being partly resistant to digestion by proteinase K under standardized conditions. It is believed to differ from PrP^C only (or mainly) conformationally, and is often considered to be the transmissible agent or prion (however, see PrP*).

● **PrP*:** Within the framework of the protein-only hypothesis, PrP* is

defined as the form of PrP constituting the essential (or only) component of the prion. It may, but need not, be identical to PrP^{Sc}.

● **'Unconventional virus' hypothesis:** Maintains that the TSE agent is a virus with unusual properties. The requirement for PrP in the host to allow replication and pathogenesis is explained by assuming that PrP forms part or all of a receptor for the virus.

These definitions describe our terminology which is, however, not agreed on by convention and is not necessarily used by others. A. A. & C. W.

from Alzheimer's to multiple sclerosis and even stroke. Activation of microglia may be pivotal in effecting neuronal damage in TSEs, dependent on the expression of PrP^C (ref. 23). But the details of this cell-death pathway and the proof that these phenomena occur in the brain during the course of the disease (and not only in Petri dishes with explanted cells) is still missing.

The march of prions to the brain

Prions are most effective when directly delivered to the brain of their host, but this is not the normal route of infection. In humans, most cases of CJD transmission were traced to intramuscular injection of prion-contaminated pituitary hormones. There is also kuru, attributed to oral uptake of infectivity during cannibalistic rituals in Papua New Guinea. Among animals, a more recent — and fateful — example is that of BSE, a common-source epidemic due to oral transmission of prions.

Remarkably, prions can find their way through the body to the brain of their host, yet histopathological changes have not been identified in organs other than the CNS. But the prions may multiply silently in 'reservoirs' during the incubation phase of TSEs. One such reservoir may be the immune system, and many studies point to the importance of prion replication in lymphoid organs. For example, type-4 PrP^{Sc} accumulates in the lymphoid tissue of tonsils

in such large amounts that it can easily be detected with antibodies on histological sections²⁴. Infectivity also accumulates in intestinal Peyer's plaques almost immediately after oral administration of prions²⁵.

The nature of the cells that support prion replication within the lymphoreticular system (LRS) is uncertain. PrP^{Sc} accumulates in the follicular dendritic cells of wild-type and nude mice (which suffer from a selective T-cell defect). However, adoptive bone-marrow transfer (which does not efficiently replace follicular dendritic cells) of wild-type cells to PrP-deficient mice restores accumulation of the infectious agent in the spleen²⁶. Immune cells are unlikely to transport the agent all the way from the LRS to the CNS; the agent probably spreads through the peripheral nervous system, as do rabies and herpesviruses²⁷. We are currently testing whether prions injected intraperitoneally may first be brought to peripheral nerve endings by cells of the LRS, with further spread occurring trans-synaptically and along fibre tracts²⁸.

Prion-resistant flocks and herds?

The discovery that mice devoid of PrP are normal, healthy and resistant to prion disease immediately suggested the possibility of generating PrP-free sheep and cattle¹⁶. This raises two questions: first, whether gene ablation can be carried out in farm animals and whether it leads to the same results as in

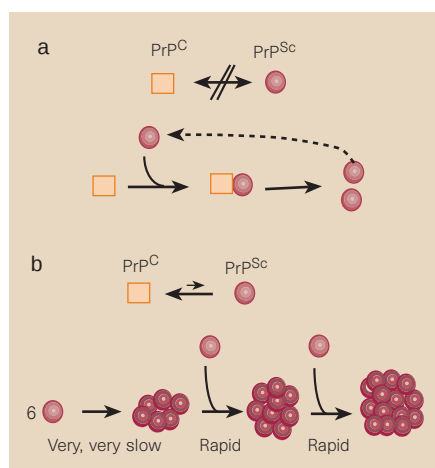


Figure 2 Models for the conformational conversion of PrP^C to PrP^{Sc}. a, The 'refolding' model. As a result of an interaction with exogenously introduced PrP^{Sc}, PrP^C is induced to change shape and form PrP^{Sc}. The conformational change is kinetically controlled — a high activation-energy barrier prevents spontaneous conversion at detectable rates. Extensive unfolding and refolding of the protein may explain the energy barrier, and the process leads to an exponential conversion cascade. With certain mutations in PrP^C, spontaneous conversion to PrP^{Sc} may be rare, explaining why familial TSEs arise spontaneously, albeit late in life. Sporadic CJD may come about through spontaneous conversion of PrP^C to PrP^{Sc}, giving rise to a conversion cascade⁹. b, The 'seeding' model. The conformational change between PrP^C and PrP^{Sc} (or a PrP^{Sc}-like molecule) is thermodynamically controlled and, therefore, reversible. PrP^{Sc} is stabilized only when it adds onto a crystal-like seed or aggregate of PrP^{Sc}. Seed formation is extremely slow, but once a seed is present monomers can add on rapidly³⁵.

mice; and second, whether this would be practical and useful.

The outbreak of BSE arose in the mid-1980s, peaked in 1992, and is now receding rapidly, mainly due to the measures taken to prevent feeding of ruminant bone and meat meal to cattle. It is virtually certain that the tragic cases of vCJD in young people are due to exposure to the BSE agent^{11,12}. Because of the long incubation times and other unknown features such as genetic predisposition and exposure criteria, we cannot assess whether the incidence of vCJD will increase, or to what extent. We need to know whether BSE will disappear completely, or whether it will become enzootic — regularly affecting animals in a particular district or season — as is scrapie, in which case it should be eradicated.

Sheep scrapie is enzootic in Great Britain, affecting 0.5–1% of the sheep population per year. Based on epidemiological data, scrapie-infected sheep are not thought to cause

disease in humans. But, in view of the oral transmissibility of BSE to sheep in an experimental setting, sheep may have been infected through feeding practices in the mid-1980s, so some cases of apparent sheep scrapie could, in fact, be due to the BSE agent. Consumption of infected sheep parts could then be a threat to human health, necessitating the elimination of scrapie.

Thus, there are circumstances in which PrP-less farm animals could be essential. The appropriate technology should be available in sheep and, probably, in cattle as well, allowing proof of principle to be established. However, the practical difficulties of replacing the many different breeds and preserving genetic heterogeneity will demand the creation and procreation of large numbers of genetically modified animals — a truly daunting enterprise. Perhaps the creation of prion-resistant animals for use as a source of pharmaceutically important products could be a commercially viable first step.

Development of drugs

By the time the first symptoms of a spongiform encephalopathy are recognized, there is considerable damage in the CNS. Although there have been only a limited number of vCJD cases so far, it is impossible to predict the future incidence. Here, post-exposure prophylaxis may provide a greater chance of success than treatment of overt clinical disease. Therefore, the availability of a non-invasive or minimally invasive and affordable mass-screening procedure is essential in case the number of BSE-derived cases increases. The isolation of a monoclonal antibody specific for PrP^{Sc} could provide an inroad towards this goal, perhaps by allowing detection in tonsil scrapings²⁴.

There are several possible targets for therapeutic strategies. If, indeed, post-developmental depletion of PrP has no deleterious consequences, reduction of PrP levels may slow down or arrest disease. In principle, this could be done using antisense oligonucleotides, although effective delivery (particularly intracerebrally) remains a tough challenge. A second goal could be to inhibit the conversion of PrP into a pathogenic conformer, possibly by interfering with molecular domains that are involved in the interaction between PrP^C, PrP* or protein X. Finally, one could disrupt or inactivate the pathogenic form of PrP, perhaps using compounds with affinity for the β -sheet-rich domains, if these indeed form part of the infectious entity.

Several substances have raised limited hope for therapy — amphotericins, sulphated polyanions, Congo red and anthracycline antibiotics. The mechanism of amphotericin action is unknown, but the other compounds are thought to bind or intercalate, and disrupt, the highly ordered

structure of PrP^{Sc}. However, the effects of all compounds tested are modest. Moreover, TSEs have not attracted major investment by the pharmaceutical industry, doubtless because they are so rare in humans, making them commercially unattractive. But in the worst case, the outbreak of BSE may considerably change the epidemiology of human TSEs.

Because the time needed to develop new drugs is decreasing, and the peak of a conjugal BSE epidemic in humans may be reached in 10–20 years, the development of therapeutic agents is both timely and mandatory. If such an effort is not undertaken by industry, we believe that a programme to identify therapeutic compounds and show proof of principle should be underwritten by the British government — politicians should consider their responsibility towards the people who have been exposed to the BSE agent. □

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Lisa's laws

Leonardo da Vinci is almost as well remembered for his scientific investigations as for his paintings and sculpture. His work as an artist was informed by his insight into science, as his system of 'natural laws' demonstrates.

Martin Kemp

To begin a series on art and science* with Leonardo da Vinci's *Mona Lisa* may seem obvious and even banal. But even such a familiar work can be scrutinised through fresh eyes if we set aside our stock categories of art and science. For Leonardo, the invention of a compelling image was not based on literal imitation of nature or unlicensed imagination but on the remaking of natural effects through the understanding of natural laws derived from 'experience'. The inventor's imaginative faculty or *fantasia* worked in concert with the intellect to recreate an infinity of plausible images.

Every painted effect was, in theory, based on a natural law. I will highlight just three of those laws that are inherent in the *Mona Lisa*. These restate in more compact form what Leonardo wrote about the phenomena of light on surfaces, the formation of spiral configurations, and the huge changes in the bones, flesh and blood in the "body of the Earth".

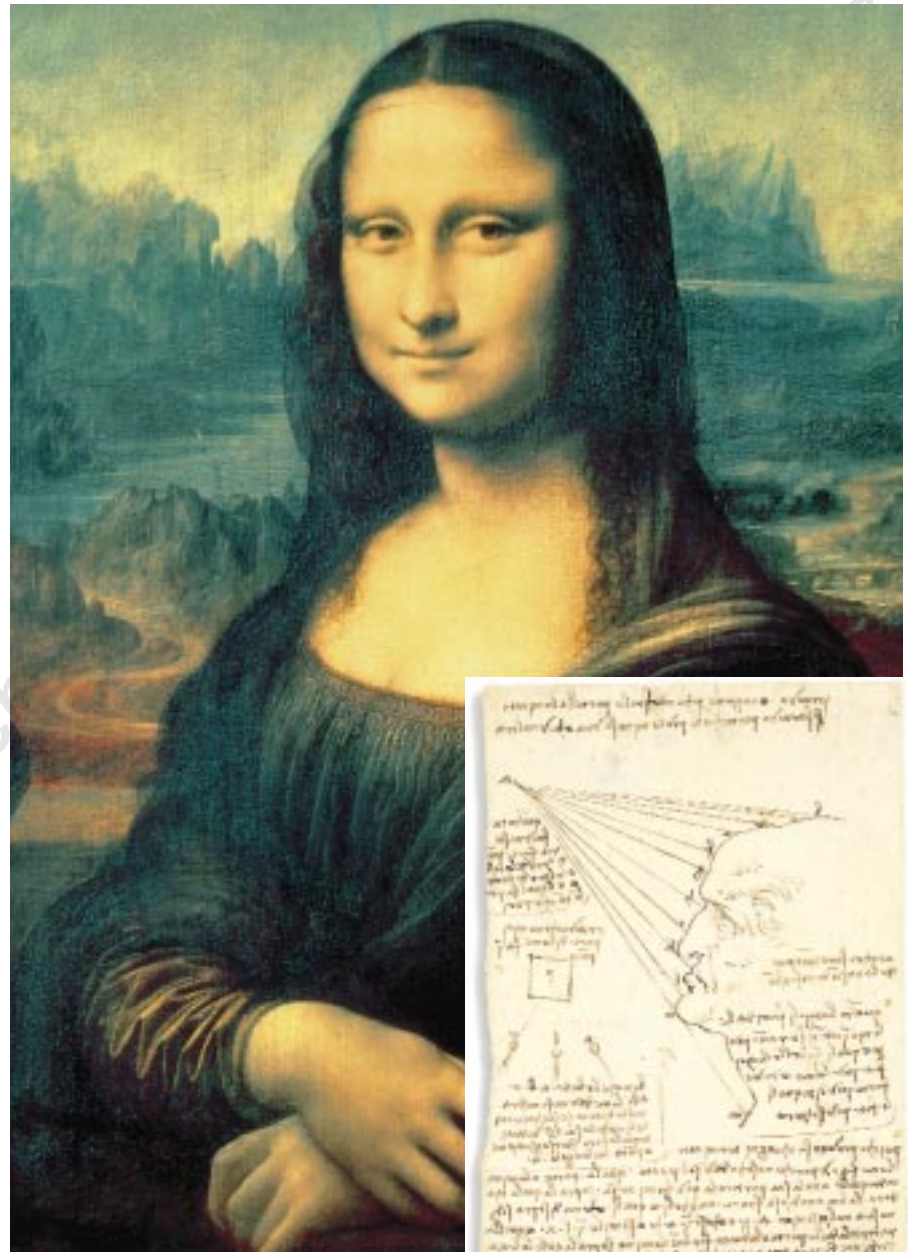
Law 1 "The intensity of light on a plane is proportional to the angle of impact of the light on that plane."

So, on a human head, light from a point source will make its strongest impact when it strikes a surface perpendicularly. When it strikes a glancing blow, the intensity of light at that point will be proportionately weaker. The relative intensities of light on any solid body must be characterized according to this rule.

In the eighteenth century Johann Heinrich Lambert established that the brightness was proportional to the cosine of the angle between the line of sight and that normal to the plane — the law now observed in computer rendering.

Law 2 "A helix arises from the combination of two components: a straight axis corresponding to a linear motion or a weight acting in a vertical direction; and a circular motion or shaping force in a plane perpendicular to that axis."

A current of air or water will exhibit a desire to move forward, just as a weight desires to fall, but will be deflected by successive impacts to induce a revolving impetus, just as a substance that has an inherent desire to curl, such as hair, will be disposed to turn around the axis of its weight. Phenomena that involve direct and revolving impulses include vortices in water currents, ringlets of hair,



gathered or compressed drapery, the growth of leaves in plants, shells of marine creatures, spiral staircases and conical gears for clocks.

Law 3 "The relative positions of the sphere of water and the 'body of the Earth' are constantly in flux, such that different zones of solid earth are extruded from the sphere of water and become subject to erosion, while the inner caverns of the Earth are also subject to periodic collapse."

So mountains are eroded at their bases, eventually collapsing and damming rivers

Shedding light on *Mona Lisa*: Leonardo da Vinci's *Demonstration of the Intensities of Light on a Human Face According to Angles of Impact* (inset).

to form lakes at different levels, the higher of which will in time burst forth, leaving stranded water creatures — as is witnessed by the deposits of ancient shells in high places. □

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*See the explanatory leading article in *Nature* 389, 213; 1997.

Sex on the brain

Reports of morphological differences between the brains of humans with different sexual orientation¹ or gender identity² have furthered speculation that such behaviours may result from hormonal or genetic influences on the developing brain. However, the causal chain may be reversed; sexual behaviour in adulthood may have caused the morphological differences. I report how adult sexual experience alters the appearance of rat motor neurons as revealed by Nissl staining, the same technique used in post-mortem human studies.

Male Sprague–Dawley rats were castrated at 117–123 days of age and I implanted 5-mm-long testosterone-filled Silastic capsules subcutaneously, to produce a much lower than normal concentration of circulating androgen while sustaining continued male copulatory behaviour³. One week later I gave each rat as a cage-mate an ovariectomized female that had been subcutaneously implanted with either a 5-mm capsule containing 10% oestradiol to induce constant behavioural receptivity⁴, or an empty capsule.

Male rats provided with oestrogen-treated cage-mates began copulating shortly after the introduction of the receptive female and were therefore labelled 'copulators'. The males caged with untreated (and therefore unreceptive) females were never observed to copulate and are designated 'non-copulators'. Females were replaced from a reserve pool every 3 days (with confirmation that only the oestrogen-treated females were receptive) for 27 days.

I stained the spinal cords from the males

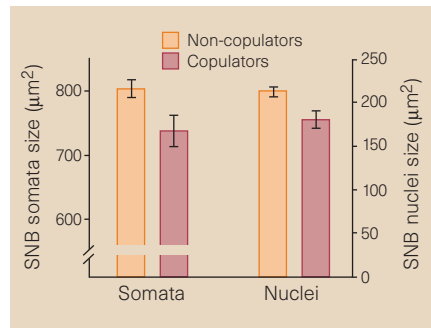


Figure 1 Sexual experience alters neuronal morphology.

with thionin to reveal motor neurons in the spinal nucleus of the bulbocavernosus (SNB)⁵. These motor neurons and their striated target muscles are active during male copulatory behaviour⁶, and shrink after castration unless replacement testosterone is provided⁷. Changes in the size of SNB somata and nuclei are accompanied by changes in the number and size of synaptic inputs to the motor neurons⁸. An observer, blind to group membership, determined the number and cross-sectional area of SNB somata and nuclei⁷.

As expected, SNB somata and nuclei were smaller than in gonadally intact males⁷ (Fig. 1), and there was no difference between the groups in the number of SNB motor neurons. The number of neurons, which is reported in only a few human studies⁹, appears stable. The ten copulator males had significantly smaller SNB somata ($P < 0.04$, two-tailed t -test) and nuclei ($P < 0.02$) than the nine non-copulators.

The bulbocavernosus muscles innervated by the SNB were also lighter in copulators (711 ± 37 mg; mean $\pm$ s.e.m.) than in non-copulators (868 ± 50 ; $P < 0.02$). The animals received equivalent androgen exposure as shown by the lack of difference in either body mass ($P > 0.20$) or the mass of the highly androgen-sensitive seminal vesicles (168.5 ± 12.9 mg, copulators; 177.8 ± 11.9 mg, non-copulators).

Copulatory experience can therefore alter the size of neurons, as revealed by Nissl staining. Whether the sensory experience or motor activity of copulation induced these morphological changes, interpretations of correlations between human behaviour and neural morphology must acknowledge that the two are reciprocally related^{10,11}. It is possible that differences in sexual behaviour cause, rather than are caused by, differences in brain structure.

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Antibiotic resistance spread in food

Nutritive and therapeutic treatment of farm animals with antibiotics, amounting to half of the world's antibiotic output, has selected for resistant bacteria that may contaminate the food produced. Antibiotic-resistant enterococci and staphylococci from animals are found in food when they survive the production processes, as in raw cured sausages and raw milk cheeses¹. The broad host ranges of some plasmids and the action of transposons in many bacteria allow antibiotic-resistance genes to be communicated by conjugation between different species and genera^{2,3}. A multi-antibiotic resistance plasmid from a lactococcus found in cheese provides a historical record of such events.

Lactococci function as starter cultures

for lactic fermentation in cheese and have conjugative 'metabolic' plasmids⁴. The streptomycin-, tetracycline- and chloramphenicol-resistant *Lactococcus lactis* strain K214 was isolated by us in 1993 from a raw milk soft cheese (2×10^8 colony-forming units (c.f.u.) per g). A circular 29,871-base-pair plasmid (pK214) transferred all three resistances to *Enterococcus faecalis* strain JH2-2 by electroporation. We determined the complete nucleotide sequence of pK214 and identified homologous molecular structures in other organisms (Table 1).

A putative *Lactococcus* region of the plasmid contains genes for a resolvase, a replication-associated protein, a DNA nickase, insertion sequence IS904 and a new insertion-sequence element. A new membrane-spanning efflux protein confers increased macrolide resistance (erythromycin and others) on *Escherichia coli*, but not on strain K214 itself. A *repB* gene and tandem repeats

(iterons) identify pK214 as a theta-type replicating plasmid.

A mainly *Staphylococcus*-derived segment contains the information for the streptomycin-inactivating adenylase, the chloramphenicol-inactivating acetylase and replication protein RepD, known from staphylococcal plasmids. A mobilization gene is homologous to a gene from a *Lactobacillus plantarum* plasmid. The region is flanked on both sides by insertion-sequence elements from *Enterococcus faecium*.

The tetracycline-resistance gene (providing ribosome protection) is 99.8% homologous to *tet(S)* from *Listeria monocytogenes*. It is joined by sequences similar to a region of transposon Tn916 from *E. faecalis* which is involved in *tet(M)* expression. Another *E. faecium* insertion-sequence element complements the assembly.

Fifteen open reading frames (ORFs) including six insertion-sequence elements aid DNA rearrangement, regulation, mobi-

Table 1 Homologies to sequences from the antibiotic-resistance plasmid pK214

ORF	Position (base pair)	Highest homology	Sequence identity (%)	Organism	GenEMBL accession number
1	21–593	Resolvase	63.6	<i>Lactococcus lactis</i>	X99798
2	681–1448	Replication-associated protein SOJ	29.1	<i>Mycoplasma</i>	V34816
3	1451–1750	Unknown	–	–	–
4	1824–2189	Unknown	–	–	–
5	2204–2584	Unknown	–	–	–
6	2610–3554	Unknown	–	–	–
7	4236–5390	Replication protein RepB	82.3	<i>Lactococcus lactis</i>	L02920
8	5868–6185	Unknown	–	–	–
9	7231–8925	DNA nickase	24.4	<i>Streptococcus agalactiae</i>	L39769
10	9206–10358	IS904 (transposase)	99.8	<i>Lactococcus lactis</i>	D00696
11	10534–11790	Macrolide efflux protein	34.6	<i>Streptococcus pyogenes</i>	U70055
12	11879–12331	phnB protein	29.9	<i>Escherichia coli</i>	J05260
13	12458–12991	Protein p35	38	<i>Escherichia coli</i>	X13270
14	13008–13382	IS904 (truncated transposase)	100	<i>Lactococcus lactis</i>	D00696
15	13439–14899	IS150 (transposase)	40.8	<i>Escherichia coli</i>	X07037
16	15678–15127	Resolvase	78.8	<i>Staphylococcus aureus</i>	X16298
17	16998–16153	Regulation protein (Rob)	40	<i>Escherichia coli</i>	M97495
18	17087–17896	IS1216	98.2	<i>Enterococcus faecium</i>	L40841
19	18908–18060	Streptomycin adenylase	98	<i>Staphylococcus aureus</i>	X06627
20	19755–18916	Replication protein RepD	89	<i>Staphylococcus aureus</i>	X02166
21	21663–20492	Mobilization protein (Mob)	59	<i>Lactobacillus plantarum</i>	M33531
22	22563–21916	Chloramphenicol acetylase	99.9	<i>Staphylococcus aureus</i>	M90091
23	23036–22698	Replication initiator (truncated)	95.1	<i>Listeria monocytogenes</i>	X68412
24	23133–23941	IS1216	98.7	<i>Enterococcus faecium</i>	L40841
25	24429–24199	ORF 7 of transposon Tn916	56.2	<i>Enterococcus faecalis</i>	L15633
26	24906–25265	ORF 9 of transposon Tn916	53.6	<i>Enterococcus faecalis</i>	L15633
27	25964–25779	ORF 6 of transposon Tn916	80.3	<i>Enterococcus faecalis</i>	L15633
28	27974–26034	Tet(S)	99.8	<i>Listeria monocytogenes</i>	L09756
29	29016–29824	IS1216	99.6	<i>Enterococcus faecium</i>	L40841

Assignments of ORFs of the *Lactococcus lactis* antibiotic-resistance plasmid pK214 (GenEMBL accession no. X92946) to known genes and functions on the basis of DNA homology scores and derived amino-acid sequences. IS, insertion sequence. The complete nucleotide sequence of pK214 was obtained by the 'primer-walking' technique applied to overlapping fragments cloned in pUC18.

lization and replication. Seven ORFs have to do with antibiotic resistance and seven are unknown, ill-defined or truncated. No known plasmid-transfer genes are present. Whether conjugative mobilization of pK214 can be triggered by the other three plasmids of strain K214 (sizes roughly 10, 20 and 50 kilobase pairs) remains to be established.

In plasmid pK214, *Lactococcus* K214 has, with the help of insertion-sequence elements, collected genetic information from four other species to construct an antibiotic survival kit that also works in *E. faecalis*. pK214 is a live record of previous genetic exchange between pathogenic and non-pathogenic bacteria in food-associated environments. It is further demonstration of the presence of transmissible antibiotic-resistance genes in the human food chain. The resistant bacteria probably originated from antibiotic treatment of the cows. As lactococci may be found together with enterococci and staphylococci as part of the cows microflora, resistance transfer and pick-up may have occurred in the animals or during cheesemaking, where enterococci, listeria and staphylococci survive and eventually multiply.

When analysed in 1991 and 1995, this

cheese brand had also contained different enterococci resistant to tetracycline, chloramphenicol, gentamycin, penicillin, erythromycin, lincomycin and vancomycin (mechanism unknown but neither vanA nor vanB) at 10^6 – 10^7 c.f.u. g^{-1} (ref. 1). This cheese was the source of a *Listeria monocytogenes* epidemic in 20 patients in 1995⁵.

To preserve the life-saving potential of antibiotics, the spread of resistance genes at all levels must be stopped⁶. Distribution routes like those between animals, food and consumers have to be interrupted. In this example, it could be achieved by using pasteurized milk for cheese making. The situation might also be helped by stopping the inappropriate use of the selective antibiotics in animal husbandry. Regarding the presence of transmissible antibiotic resistances in microorganisms from food, a rethinking of food safety issues has recently been proposed⁷.

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Targeted disruption in *Arabidopsis*

Homologous recombination has been used for two decades to target insertions into cloned genes in bacteria and yeast, and more recently has become a routine method of gene inactivation in mammals. *Arabidopsis* is one of several multicellular model organisms (along with *Drosophila*, *Caenorhabditis* and zebrafish) in which mechanisms controlling development have been studied. Previously, traditional genetic methods have been used, as targeted disruption by homologous recombination has not been successful in any of these organisms. We have now successfully disrupted the AGL5 MADS-box gene in *Arabidopsis* by homologous recombination, providing a useful tool for future analyses.

Experiments in cultured *Arabidopsis* cells using a root transformation procedure have indicated that targeted disruption of cloned genes should be possible¹. But regeneration was not possible from this cell line, prohibiting the isolation of homozy-

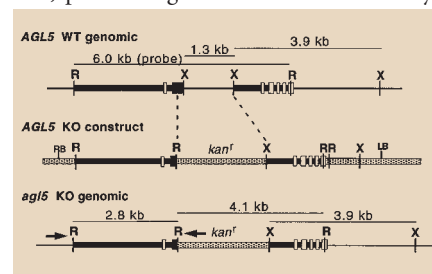


Figure 1 Diagram of wild-type AGL5 genomic region, knockout (KO) construct, and *agl5* mutant genomic region. Thick black lines indicate the genomic region included in the targeting construct. Dashed lines indicate the genomic region that was targeted for replacement by the kanamycin-resistance cassette (*kan^r*). Also shown are the positions of oligonucleotide primers (arrows) used to identify the targeted insertion, and the probe used in DNA analyses. Exons are indicated by white boxes, the black box represents the MADS-box, and the stippled bar represents the pZM104A vector¹ including the *Agrobacterium* left (LB) and right (RB) T-DNA borders. R, EcoRI; X, XbaI.

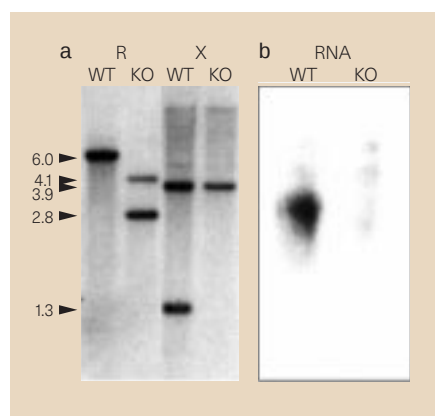


Figure 2 Genetic analysis of *agl5* mutant. **a**, Genomic DNA blot with 6.0 kb *EcoRI* fragment as probe. The 3.9-kb *XbaI* fragment was present in wild-type and mutant plants, whereas the 1.3-kb *XbaI* fragment was present only in the wild type. This same probe hybridized to a 6.0-kb *EcoRI* fragment in the wild type and to the expected 4.1 and 2.8-kb *EcoRI* fragments in the mutant. **b**, A probe specific for the 3' end of the *AGL5* complementary DNA detected the *AGL5* transcript in wild-type but not in the knockout mutant plants. Details of methodology available from the authors on request.

gous mutant plants. Recent improvements in intact plant transformation technology² have provided a more efficient method for generating large numbers of transgenic plants, so that it should be possible to identify a targeted insertion, even if the frequency of such an event is relatively low.

We used a polymerase chain reaction (PCR)-based assay of transgenic plants to identify targeted insertions into *AGL5*^{3,4}. The targeting construct consisted of a kanamycin-resistance cassette that was inserted between a roughly 3-kilobase (kb) segment from the 5' end of the *AGL5* gene and a 2-kb segment at the 3' end (Fig. 1). We produced 750 kanamycin-resistant transgenic lines by *Agrobacterium*-mediated transformation, and analysed pools of transformants using PCR to determine whether any of these primary transformants contained the desired insertion into *AGL5*. We found a single line that seemed to contain the anticipated insertion, and this line was allowed to self-pollinate to permit further analyses in subsequent generations.

We analysed genomic DNA from the homozygous mutant plants with five different restriction enzymes and using several distinct PCR amplifications. All data were consistent with the desired targeting event (Fig. 2a and data not shown). Additional genomic DNA analyses and PCR amplifications confirmed that there were no detectable deletions and/or rearrangements outside the targeted region. As expected, we detected the *AGL5* transcript in wild-type but not in *agl5* mutant plants (Fig. 2b).

Targeted disruption by homologous recombination provides a new tool along

with other methods, such as T-DNA insertion and transposon tagging, for obtaining loss-of-function (knockout) alleles of cloned genes in *Arabidopsis*. Homologous recombination has a number of advantages over these methods, being a relatively rapid procedure (roughly six weeks from the time *Arabidopsis* plants are transformed to the time that a targeted disruption can be identified) that allows precise construction of both knockout and more subtle 'knockin' mutant alleles⁵.

Although we have not yet determined the precise frequency of homologous recombination, continued improvements in plant transformation technology and the use of negative selection strategies suggest that targeted disruption will become a routine tool for molecular and genetic studies of *Arabidopsis*. This is now the only higher eukaryote in which both large-scale random mutagenesis and targeted disruption by homologous recombination are feasible.

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Speciation in the open ocean

Species inhabiting the open oceans generally occupy broad distribution ranges, extending from parts of ocean basins to entire oceans¹. Such oceanic species are accepted as monospecific because of their tendency to be dispersed across great distances by ocean currents. Indeed, there are few known examples of cryptic diversity among species in the oceanic environment². We have now found large, localized genetic differences within a circumglobal, monotypic species of deep-sea fish. This shows that cryptic allopatric lineages can and have split without discernible barriers, suggesting that there might have been a serious underestimation of oceanic biological diversity.

We studied a species of the deep-sea fish genus *Cyclothone* (family Gonostomatidae) that comprises 13 oceanic micronektonic species³ (20–70 mm body length). They are so ubiquitous and numerically dominant in meso- and bathypelagic depths (200–2,000 m) in the world's oceans that some authors have claimed them to be the most abundant vertebrates on Earth⁴. Taxonomic confidence in *Cyclothone* has been achieved through several critical revisions using worldwide collections³, and mitochondrial DNA sequence data have supported the recognition of the 13 species⁵.

We sequenced a portion of the 5' half of the mitochondrial 16S ribosomal RNA and adjacent transfer RNA^{val} genes (366 base pairs) from 41 specimens of *Cyclothone alba*, which occurs circumglobally along tropical–subtropical latitudes at depths of 300–600 m (Fig. 1), in addition to seven specimens of *C. signata*, four of *C. braueri* and one of *C. acclidensis*, and analysed the unambiguously aligned sequences (350 base pairs) using neighbour-joining and maximum parsimony methods (Fig. 1).

There is complete congruence between genealogy and geography in this phylogeny. Each individual belonged to one of five monophyletic groups (bootstrap probabilities 91–100%) that corresponded to the region of its origin (Fig. 1). The extant sister populations seem to have undergone relatively long, independent lineage-sorting processes and attained reciprocal monophyletic status⁶ with low levels of gene flow⁷, but no discernible morphological divergence. Indeed, the maximum intraspecific uncorrected pairwise sequence difference in *C. alba* (11.1%) was very similar to the minimum interspecific differences between *C. alba* and *C. signata* (11.9%), the average intra- and interpopulation sequence differences of *C. alba* ranging from 0.1 to 0.7% and from 3.0 to 10.3%, respectively.

The central and western North Pacific populations are more closely related to the western North Atlantic (bootstrap probabilities, 96%/99%) and eastern equatorial Indian Ocean populations (bootstrap probability, 93%/92%), respectively, than to the other Pacific populations. The existence of two such inter-oceanic ancestral populations can be explained by the fact that tropical Pacific and Atlantic waters had been connected through the Panamanian seaway until the final closure of the isthmus about 3.2–2.8 million years ago⁸ and that Pacific and Indian deep waters have been narrowly connected through the Strait of Timor in the equatorial area. Subsequent lineage splittings may have resulted from the closure of the isthmus and the Indonesian Archipelago acting as effective geographical barriers.

There seem to have been earlier lineage splittings of the western North Atlantic and central North Pacific populations, the west-

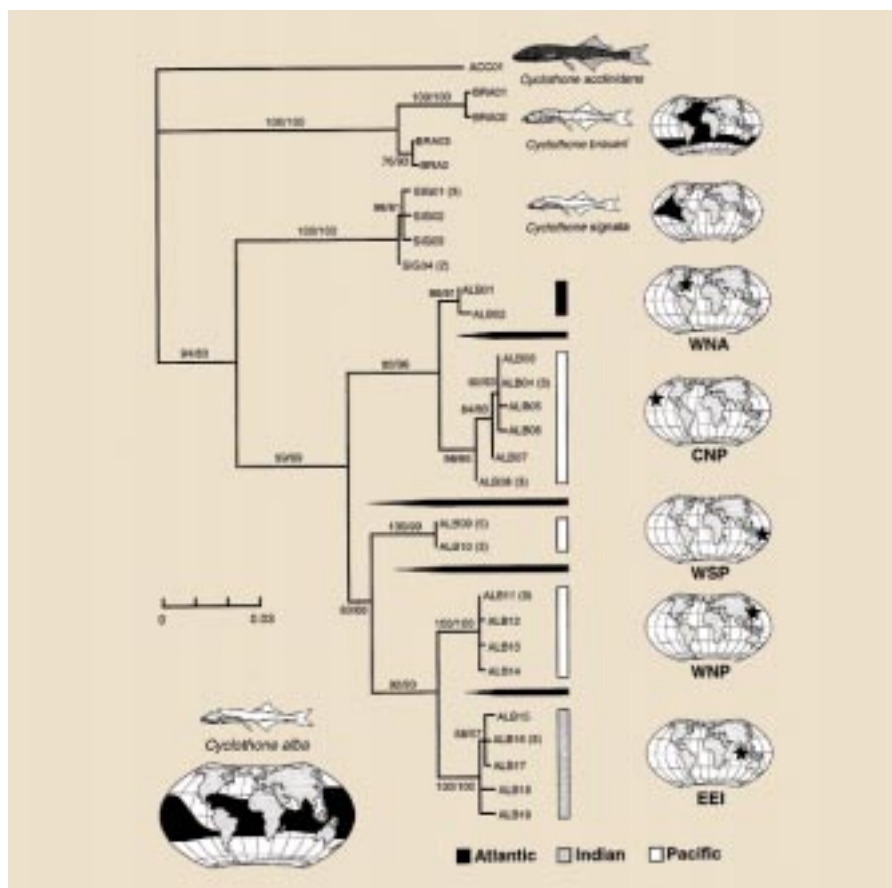


Figure 1 Neighbour-joining tree of the four species of *Cyclothone*. Tree topology is congruent with one of the two maximum parsimony trees (186 steps; consistency index = 0.742; retention index = 0.924). Pairwise distances were calculated using the Kimura two-parameter method, and neighbour joining analyses used MEGA¹⁰. Maximum parsimony analyses used the heuristic algorithm in PAUP¹¹, with no transition or transversion weightings. Phylogenetically uninformative sites were excluded. Haplotypes are designated by the first three letters of specific names and two digits. Number of individuals (if any) with identical sequences follows in parentheses. Numbers beside internal branches indicate bootstrap probabilities¹² (> 50% only) for 1,000 replicates. Wedge-shapes denote possible population subdivision events. Shaded portions of maps show distribution ranges⁵. Specimens were taken from two stations in the Sargasso Sea (western North Atlantic, WNA), two in Hawaiian waters (central North Pacific, CNP), two in the equatorial eastern Indian Ocean (EEI), one in the Coral Sea (western South Pacific, WSP) and three off southern Japan (western North Pacific, WNP).

ern South Pacific population, and the western North Pacific and eastern equatorial Indian Ocean populations, which required three within-ocean fragmentations of the ancestral population in the Pacific (Fig. 1). It is evident that these three lineages have not been ephemeral, as there has been a lack of within-ocean coalescence between populations during the past few million years, which probably extends back beyond the closure of the Panamanian seaway.

Our results show that there are genetically structured, isolated populations in open oceans. Given the long periods where there were no physical barriers, such as continents, it seems surprising that the three populations in the Pacific have not coalesced. Numerous questions remain unanswered, such as what factors might structure genetic diversity in such a homogeneous environment. As stated by Gibbs⁹, we are a long way from knowing what species really exist in the oceanic pelagic realm.

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Extraterrestrial handedness

M. H. Engel and S. A. Macko¹ tentatively ascribe the enantiomeric excess of L-amino acids in extraterrestrial sources to the circular polarization of synchrotron radiation, from a neutron star, incident on the interstellar molecular cloud from which the Solar System formed (see also the accompanying News and Views article by C. F. Chyba²). But the Kuhn–Condon zero-sum rule^{3,4} for the rotational strengths of a chiral molecule requires that broad-band circularly polarized radiation cannot discriminate between the enantiomers of a racemic substance in photochemical reactions.

Kuhn first demonstrated photochemical optical resolution by showing that monochromatic circularly polarized light of a given handedness, tuned to the frequency of a specific circular dichroism absorption of an enantiomer, preferentially photolysed that enantiomer in a racemic mixture³. The mirror-image enantiomer was favoured if the radiation was tuned to a circular dichroism absorption of opposite sign, under the same conditions. Kuhn used coupled-oscillator theory to account for the observation that the circular dichroism bands of an enantiomer alternate in sign along the wavelength ordinate, and so cancel out over its electronic spectrum. Therefore the optical rotatory power of a given enantiomer, and its susceptibility to differential photochemical change with circular radiation, sum to zero over the electromagnetic spectrum as a whole³. Kuhn's classical result was confirmed by Condon⁴, who demonstrated quantum-mechanically that an enantiomer's rotational strengths (measured by the circular dichroism band areas) sum to zero over the spectrum.

The zero-sum rule does not exclude a photochemical origin for biomolecular homochirality under severely restrictive initial conditions, covering time, place, radiation filters, and so on, for example by a prebiotic pool of racemic amino acids on an east-facing slope exposed to solar radiation only at dawn on the early Earth⁵. But such suggestions are essentially *ad hoc*.

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Bringing order to mental disorders

Making Us Crazy: DSM — The Psychiatric Bible and the Creation of Mental Disorders

by Herb Kutchins and Stuart A. Kirk
The Free Press: 1997. Pp. 293. \$27.50

Roy Porter

Thanks to its air of high farce, one episode in modern psychiatry has assumed legendary status. In a postal ballot in 1974, members of the American Psychiatric Association (APA) voted, by a rather slim margin, to delete homosexuality from its catalogue of mental disorders.

The poll followed frenzied and sometimes violent lobbying: the association's conventions had been stormed by Gay Lib activists — they should be shot, one participant demanded. And a conference had been addressed by a cloaked and hooded 'Dr Anonymous', who declared himself gay and proceeded to disclose that more than 200 fellow members of the association were also homosexual, thereby in effect threatening to 'out' the closet gays.

This notorious affair so smacks of pantomime — the abolition of a major 'psychiatric disorder' by majority vote — that it is tempting to assume it must have been a one-off event, uniquely scandalous in its implications. But the message of *Making Us Crazy* is the reverse: the whole history of the *Diagnostic and Statistical Manual (DSM)*, published by the APA since 1952, has been one of non-stop wheeling and dealing, the only difference being that the diagnostic horse-trading has usually taken place not in the full glare of publicity but behind closed doors. Herb Kutchins and Stuart Kirk bring the politics of disease-naming out into the open.

DSM started small — the first edition was a bare hundred pages — but it just grew and grew, the latest version, *DSM-IV*, issued in 1994, running to 900 pages and 300 disorders. *DSM* became the professional bible because it proved a godsend. To a profession deeply factionalized and hopelessly incapable of agreeing on either theories or therapies, descriptive diagnostic categories offered at least the appearance of scientific objectivity and hence consensus. Moreover a disease labelled looks like a disease half-conquered.

And over the years the business of diagnosis took on greater practical consequence: a patient with an authorized *DSM*-coded diagnosis is one whose treatment can be billed to insurers, health maintenance organizations or federal bodies. Moreover, it was found that two could play the diagnostics game, with user lobbies learning to demand the right to a disease tag to legitimize misery and file claims for compensation.

With the stakes thus raised, much hinged upon the inclusion or exclusion of a particular label in the next *DSM*. It is this 'gatekeeper politics' that Kutchins and Kirk explore, concentrating on half a dozen syndromes. At the centre of their story is the Columbia University psychiatrist Robert Spitzer, for long the impresario and diplomat of the *Manual*, seeing it through successive editions on the basis of an unswerving insistence that *DSM* stands for rigorous scientific objectivity.

Yet diagnostic terms have come and gone like summer fashions. The most spectacular exit was homosexuality — though not without the concoction of spurious diagnostic categories such as "ego-dystonic homosexuality" as a compromise patched up between radicals and conservatives.

The most striking new arrival has been post-traumatic stress disorder (PTSD). Framed in the wake of the Vietnam war, PTSD made a bashful first appearance in *DSM-III* in 1980 and then enjoyed quite unanticipated popularity, spreading from battle victims to all manner of alleged victims of family emotional and sexual abuse, after it was hijacked by users' groups with the collusion of sympathetic or opportunistic practitioners.

In some cases the fate of a candidate diagnostic category has hung in the balance. In drafts for the revised *DSM-III* a new entity called "masochistic personality disorder" was given an airing. Sufferers were said to display personalities disposed to make people angry and to forgo pleasures in a quite

abnormal manner. Rightly suspecting that the APA committee identified this as a woman's complaint, feminists exposed the diagnosis as a none-too-subtle way of transferring blame to the victims of abusive husbands and lovers.

Feminist psychiatrists then turned the tables by coming up with a mirror-image diagnosis for men: "delusional dominating personality disorder". The committee — almost all male, with the exception of Spitzer's wife! — found this riposte hard to stomach. After various shifty expedients, including renaming it "self-defeating personality disorder", the masochist label was binned.

Nevertheless, as Kutchins and Kirk note, one doesn't have to look far in the latest *DSM* to find signs of abiding gender and race bias, and other tell-tale prejudices.

Making Us Crazy challenges the APA's claim that *DSM*'s diagnostics simply reflect the facts. On the contrary, its categories have been 'constructed', through the exercise of professional influence. The conclusion reached by the Cambridge psychiatrist G. E. Berrios in his magisterial *History of Mental Symptoms* (Cambridge University Press, 1996) — that there is no such thing as an

Masked Baule

The striking masks made by the Baule people of the Ivory Coast are increasingly familiar objects in Western art galleries and museums. In *Baule: African Art, Western Eyes* (Yale University Press, \$60, £30), Susan Mullin Vogel uses 25-years' experience of living and working with the Baule to explore both the Western perception of the art and the use of the sculptures by the Baule people themselves. The book, containing many stunning photographs of both art and everyday life in West Africa, shows that the masks are part of a wider sculpture tradition embracing artefacts of many kinds. And though some examples of the art, such as this Mbolo rabbit mask, now in a Belgian collection, are everyday items in Baule life, others have a darker significance in which display plays no part.



atheoretical diagnostic language — is thus given further support.

While denying that they are ‘anti-psychiatry’, Kutchins and Kirk do deplore the proliferation of psychiatric labelling, facilitated by *DSM*’s ever-lengthening diagnostic list. Nowadays many groups, and not just psychiatrists, patently have an interest in translating everyday behaviours into psychiatric diseases — worry for example becomes “generalized anxiety disorder”.

In this medicalization process, the wretched and the powerless are all too easily further victimized by labels that carry a lasting stigma. One solution, of course, would be for the public acceptance, without shame, of mental disorder. But that would be crying for the moon.

This is a serious and well-documented study, which casts serious doubt on the touted scientific status of *DSM* categories. It is also readable, although Kutchins and Kirk’s preoccupation with the day-to-day minutiae of the politics of naming may dispose some psychiatrists to see in this a case of ancient obsessional disorder. It is certainly sobering to discover just how the terms we take for truth have come into currency. □

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Where has the billion trillion gone?

The Conscious Universe: The Scientific Truth of Psychic Phenomena
by Dean I. Radin

HarperEdge: 1997. Pp. 320. \$25.

I. J. Good

My friend Christopher R. Evans worked for a time with the well-known parapsychologist J. B. Rhine, but became a sceptic. In 1974 I invited Evans to Blacksburg, Virginia, to give a lecture on extra-sensory perception (ESP), and picked him up at Roanoke airport. He had travelled from London in a Boeing 727. The licence number of my car happened to be CRE 727. The probability of that coincidence was about $1/26^3 \times 1,000 \approx 1/17,000,000$.

I have experienced three even more remarkable coincidences, one of which changed the course of my life. But I doubt whether these coincidences were paranormal because there are more than 5 million minutes a decade. Some people must have experienced chance coincidences with probabilities of about 10^{-14} . So controlled, not anecdotal, observations are needed.

In England, for about 20 years starting in 1939, S. G. Soal was by far the most promi-



Body language

Facial features of the sanguine, phlegmatic, melancholic and choleric personality types (clockwise from top left), taken from Johann Lavater’s *Essays on Physiognomy* (1789). They are reproduced in *Believing in Magic: The Psychology of Superstition* by Stuart A. Vyse

(Oxford University Press, £18.99, \$25). Vyse argues that scientific analysis of differences in personality traits — such as sensitivity to coincidence, fear of failure, a need for control — can help us to understand why superstition and belief in the paranormal are so prevalent today.

nent parapsychologist. He did controlled card-guessing experiments resembling those of Rhine. At the suggestion of Whateley Carington, Soal examined his records, looking for ‘hits’ one ahead and one behind the guess of the ‘current’ card. In one series of experiments, the tail probability, or *P* value (the probability that, by chance, the outcome would be at least as ‘extreme’ as the observed outcome), was 10^{-35} for the ‘one-aheads’, thus seeming to prove the existence of pre-cognitive telepathy.

But evidence accumulated, culminating in the ingenious detective work of Betty Markwick in 1978, showing that Soal’s studies were very probably fraudulent. Dean Radin, author of *The Conscious Universe*, avoids mentioning Soal.

Radin is firmly convinced that paranormal events happen. His conviction is based mainly on evidence from controlled experiments but is influenced also by the ‘non-local’ phenomena of quantum mechanics.

Quantum mechanics has affected many people’s metaphysical speculations about consciousness and ESP. For example, some 50 years ago, in a conversation with the prominent physicist Léon Rosenfeld about subjective experiences, I said: “A [quantum]

field theory does seem to be natural in order to understand how the activities of numerous neurons in a brain somehow summate. Perhaps psi depends on ψ [the Schrödinger wave function].”

Leaving psi aside, there are much more serious and technical speculations about the relationship between consciousness and quantum fields by Stuart Hameroff and Roger Penrose, related to microtubules — extremely small skeletal elements in neurons. One could say that microtubules update Descartes’ pineal gland. Penrose does not, however, mention ESP in his work on consciousness.

Taken at its face value, some of the evidence from controlled experiments is conclusive. But we have to allow for fraud and the ‘file-drawer’ effect. Take the first of these. Even many ‘normal’ scientists have cheated, as recorded by Alexander Kohn in *False Prophets: Fraud and Error in Science and Medicine* (Barnes and Noble, 1986). To that collection may be added the psychologists who lie to their subjects and call the lying ‘experimental dissimulation’.

Parapsychologists and psychics have more incentive to cheat because, if their research results are uninteresting, they have

less opportunity to turn to teaching. Unconscious cheating, wishful thinking (which is universal), unsound experimental design and analysis, and seeing what we expect are further pitfalls. The statistician M. G. Kendall once described the phenomenon of seeing what one expects as "one of the deadliest forms of bias in psychology". He was referring to an experiment in which an observer of a reliable random-number generator had a tendency to write down too many even numbers.

Potentially the most important evidence in Radin's book is in the chapter on meta-analysis, which is also emphasized in the introduction — and it is here that the 'file-drawer' effect comes into play.

Meta-analysis is the combination of results from many experiments. A problem in meta-analysis, and in statistics generally, is how to allow for the researches that remain unpublished and unknown because their *P* values did not reach a conventional significance level such as 0.05. I do not know who coined the name 'file-drawer' effect for this problem. This effect drags down the statistical significance of published work. Radin claims that "parapsychologists were among the first to become sensitive to this problem" — although he does not say when — and he mentions that "in 1975 the Parapsychological Association's officers adopted a policy opposing the selective reporting of positive outcomes". The problem was known to statisticians by 1958.

Consider the following typical example. Radin points out that there were 186 publications on ESP card tests worldwide from 1882 to 1939. "The combined results of this four-million trial database [taken at face value]," he says, "translate into tremendous odds against chance — more than a billion trillion to one." (A 'trial' is the guess of one card.) He means that the *P* value is about 10^{-21} — he is not writing only for the scientific establishment. This *P* value corresponds to a bulge above 'chance' expectation of 9.5σ , where σ is the standard deviation. (I call that a 'sigmage' of 9.5.)

Apart from the possibility of conscious and unconscious fraud and wishful thinking in some fraction of the publications, Radin claims, with no explanation, that, in order to "nullify" the statistical significance, the file drawer would have to contain "more than 3,300 unpublished, unsuccessful reports for each published report". That number 3,300 is a gross overestimate. It should be reduced at least to about 15 (or even to 8).

The expected sigmage in the file drawer, under the null hypothesis, would be slightly negative but I will call it zero. If these results were combined with the published work, the total sample size would be multiplied by 16, thus becoming 64 million individual guesses. Given the null hypothesis ('chance'), the bulge would be unaffected so

the sigmage would be divided by $\sqrt{16} = 4$ and would become $9.5/4 = 2.4$, with a *P* value of about 1/100.

Because the number of individual guesses is so large, this *P* value appreciably supports the null hypothesis (no ESP). This is because a Bayes factor (the factor by which the odds of a hypothesis are multiplied in the light of the observations), corresponding to a fixed *P* value, is roughly proportional to $1/\sqrt{N}$, where *N* is the sample size. So Radin's method for evaluating the file-drawer effect, whatever that method may be, must be misguided. This conclusion largely undermines Radin's meta-analysis which is central to his case for ESP.

Nevertheless, Radin's book is well written and provides a good summary of the arguments supporting the existence of ESP, with about 600 references. It is less good on the counter-arguments. Readers should also consult *ESP and Parapsychology: A Critical Evaluation* by C. E. M. Hansel (Buffalo, 1980), where much fraudulent work is exposed. Radin quotes Hansel as saying that three *P* values, each of 0.01, amount to one of 10^{-6} , and that he (Radin) would find that convincing. But the product of independent *P* values is not a *P* value. The product has to be transformed by a method due to R. A. Fisher. Both Hansel and Radin have overlooked this. In the present example, the composite *P* value is 1/9,000, not 1/1,000,000.

I am not a sceptic by definition. There is one type of experiment that could convince me if it were successful. Guesses, by psychics, of the parities (even or odd) of future cricket scores could be published on the World Wide Web. The actual scores and parities could be published (later) in large print to help the

precognizing of the psychics and to help their evaluation. This procedure would rule out the possibility of undetectable fraud. □

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Dangerous liaisons

Menachem's Seed

by Carl Djerassi

University of Georgia Press: 1997. Pp. 196.

\$21.95

Jack Cohen

This is the third novel in a series of what the chemist Carl Djerassi calls "science-in-fiction" in which everything mentioned could or does exist. "Most of my characters, fictional as well as real, are scientists," he says. "By exposing their lives, I try to make comprehensible the culture and behaviour of scientists."

There is a similar, specialized genre on the fringes of modern science fiction — stories about fictional scientists like fictional detectives or even fictional cowboys. Carl Sagan's *Contact* is a well known example, but Gregory Benford's *Timescape* or *Artifact*, Greg Bear's *Blood Music*, as well as Nigel Kneale's older *Quatermass* series or even E. E. 'Doc' Smith's archaic *Lensman* all come to mind as fiction about scientists. Science-in-fiction, however, seems to be less exciting and to have fewer possibilities.

I reviewed elsewhere the first of Djerassi's novels, *Cantor's Dilemma*, and recommended it, | mainstream

Toy review Smaller, cheaper, more plasticky

With Christmas still months away, and the latest Mars landing already fading in the memory, it is a good time to draw your attention to this scientific stocking-filler. In collaboration with the US Jet Propulsion Laboratory, Mattel has brought out the "Mattel Hot Wheels JPL Sojourner Mars Rover Action Pack Set".

This is a set of three small plastic models: the Pathfinder spacecraft in transit, with removable heat shield and tiny lander; a larger-scale version of the lander, with foldable panels and removable rover; and a larger-scale-still Sojourner rover. The rover is undoubtedly the star, with sprung 'rocker-bogey' suspension that allows it to take up all sorts of cute rock-sniffing postures.

The *Nature* review copy of the Mattel Hot Wheels JPL Sojourner Mars Rover Action Pack Set has now completed its primary mission objectives, successfully demonstrating the technology involved in operating for several weeks on top of a computer screen in a harsh environment of editorial curiosity, without suffering any appreciable reduction in its

operational parameters. And at \$5 initial outlay (only available in the United States, at present it is certainly in keeping with NASA's new economical philosophy.



Toy robot: the Sojourner rover model (slightly smaller than actual size).

But for those who hark back longingly to the old days of expensive, 'gold-plated' missions, an expensive, literally gold-plated model of Sojourner is available (for \$49.99). It "is mounted on a silver base plate molded to resemble the martian surface and has a handsome brass identification plate". Surely a must for every mantelpiece.

Stephen Battersby is an assistant editor of *Nature*.

time the science is much closer to my own expertise (human reproductive technology, especially intracytoplasmic sperm insertion) and I am familiar with many of the locations and personality types; I looked forward to recognizing more of the events, and expected more action.

On the surface, this is a lubricious novel about an affair between an Israeli, Menachem Dvir, formerly an atomic engineer and physicist but now a research administrator at Ben-Gurion University in Beersheba, and an American widow, Melanie Laidlaw, a grant administrator for a foundation wishing to promote sophisticated male contraception.

Conforming to the stereotype of the 'able woman administrator', she is apparently attracted to him only because she suspects most men of wanting grant-money; he, on the other hand, has not heard of her foundation and is hooked by her charms alone.

Thoughtful flashbacks that illuminate the story are recounted in pages of italics, with occasional lapses into didacticism: her masturbatory history and his sexual anatomy are relished in explicit descriptions more informative than any genuine stream-of-consciousness could possibly be. There is some excuse, however: such science as there is concerns the involvement of nitric oxide in penile erection, and the plot climax involves her secretly stealing his sperm for her own fertilization. I can't tell whether a physicist or a florist would follow the science, but on the whole it is accurate and sets the scene convincingly.

Much of the drama takes place at a fictional series of "Kirchberg Conferences on Science and World Affairs", explicitly modelled on the Pugwash meetings. This gives Djerassi the chance to evoke the early days of Ben-Gurion University and its relation to Dimona atomic research, and to portray a Palestinian scientist's progressive interaction with Menachem against the background of atomic power and weapons in the recent Middle East.

The descriptions of cameo parts are also good, and personal vignettes scattered across the broad canvas are effective in adding colour. The writing is authoritative, evocative but spare; conversation is frequently stilted — but probably justifiably so considering the subject matter being discussed.

There are a few surprising technical and linguistic infelicities: she drops his semen directly into a Dewar flask of liquid nitrogen, without cryoprotectant; an internationally famous French scientist fails to translate *mauvais nouvelles* as bad news; and I can't quite believe that a New England society matron, at a fund-raising dinner in Boston, would use the word 'prick' in the way it is used here. Worst, Menachem's reason for not wanting a child — he has been exposed to heavy radiation — has important implica-

tions even if an apparently normal spermatozoan is used for fertilization. But Djerassi ignores these.

Scientists active on the conference circuit will probably find the portrayal here true to life, and also learn about Israel and human reproduction — although not much about how to conduct an affair. Scientists who resent not being asked to such conferences will have their prejudices confirmed — all drinking and politics.

In an afterword, Djerassi says he tried hard to create an authentic ambience. He has succeeded, but that does not necessarily make the novel entertaining or mean that it will enhance the public image of science or scientists. Indeed, the whole package is rather too predictable, including the cover showing Leonardo da Vinci's *Cotillon of Hemisected Man and Woman*. Even though scientists in science fiction have a tendency to blow up the world in exciting ways, their lives are a lot more fun. □

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Sun, sea and life

Aquatic Photosynthesis

by Paul G. Falkowski and John A. Raven
Blackwell Science: 1997. Pp. 375. £39.50, \$54.95

Egil Sakshaug

"Terrestrial plants are so much part of the human experience that aquatic photosynthetic organisms are often overlooked," say the authors. It may be added that the great diversity of pigment composition and physiological properties among aquatic photosynthetic organisms, together with the fact that they are responsible for about 40 per cent of Earth's net primary production, more than justifies publication of this book.

The authors succeed in their aim of integrating knowledge from biophysics, biochemistry and physiology and applying it in an ecological and evolutionary context.

An introductory account, the book presents a wide range of topics: the introduction deals with basic photosynthetic chemistry, evolution and diversity of algae, structural and chemical properties of photosynthetic cells and useful information on quantum aspects of light, electron-spin states, energy transfer in photosystems, fluorescence and much more. The bulk of the book deals with photosynthesis proper, photosynthetic models, respiration and biosynthesis. And there are concluding chapters on global primary production, its modelling on the basis of satellite measurements of chlorophyll *a*, and the role of photosynthesis in biogeochemical cycles and past and present global change.



Marine algae: highly productive *en masse*.

The emphasis of the book is on marine photosynthesis, as reflected in the many references to the authors' own excellent studies. But photosynthesis in freshwater algae does not differ essentially from that in marine algae. Planktonic algae, including cyanobacteria, are given broad coverage whereas seaweeds are treated cursorily. Errors are immaterial and few and far between. A rare exception is the description of light-harvesting complexes in peridinin-containing dinoflagellates, which is incorrect.

Complex phenomena are admirably well described, the layout is appetizing and the illustrations instructive. The historical background is well covered, and numerous footnotes offer anecdotes and facts that make vivid reading. How many biologists know that the term 'photon' was coined by the famous chemist Gilbert Lewis and that Lord Rayleigh developed his theory of light-scattering while travelling down the Nile on honeymoon?

This enjoyable book is recommended to anyone with a general scientific background and an interest in aquatic photosynthesis and its role in biogeochemical cycles and climate. Students who specialize in aquatic photosynthesis should supplement this excellent work with texts on aquatic optics and general plant physiology. □

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Transactivation of *Igf2* in a mouse model of Beckwith–Wiedemann syndrome

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The gene *IGF2*, which encodes a fetal insulin-like growth factor, is imprinted, so only one of two parental copies of the gene is expressed. The altered expression of *IGF2* has been implicated in Beckwith–Wiedemann syndrome, a human fetal overgrowth syndrome, which is characterized by overgrowth of several organs and an increased risk of developing childhood tumours. We have introduced *Igf2* transgenes into the mouse genome by using embryonic stem cells, which leads to transactivation of the endogenous *Igf2* gene. The consequent overexpression of *Igf2* results in most of the symptoms of Beckwith–Wiedemann syndrome, including prenatal overgrowth, polyhydramnios, fetal and neonatal lethality, disproportionate organ overgrowth including tongue enlargement, and skeletal abnormalities. These phenotypes establish *Igf2* overexpression as a key determinant of Beckwith–Wiedemann syndrome.

Genomic imprinting is an important mechanism of gene regulation in which only one of the two parental copies of a gene is expressed. One of the imprinted genes, the paternally expressed insulin-like growth factor-2 (*Igf2*) gene, encodes an important fetal growth factor^{1,2}. *Igf2* is part of a cluster of imprinted genes including *Ins2*, *H19*, *Mash2*, *p57^{Kip2}*, and possibly others, which are located on distal chromosome 7 in the mouse, with the syntenic region on human chromosome 11p15.5 (ref. 3). The regulation of *Igf2* imprinting is complex and involves the neighbouring maternally expressed *H19* gene, the *H19* enhancers, and possibly other factors^{4–10}.

Imprinting mistakes are implicated in a number of genetic disorders in humans. Indeed, loss of imprinting or otherwise increased expression of *IGF2* is involved in several growth disorders and tumours¹¹. Many cancers express *IGF2* biallelically, although a causal relationship between *IGF2* dosage and tumour development has been established only in some mouse models^{12–14}.

In Beckwith–Wiedemann syndrome (BWS), there is circumstantial evidence for an involvement of *IGF2* deregulation, but demonstration of a causal relationship between *IGF2* overexpression and the symptoms is lacking. BWS is characterized by pre- and postnatal overgrowth, multiple organ overgrowth including macroglossia (large tongue), and increased risk of developing childhood tumours¹⁵. Loss of imprinting of *IGF2* with consequent biallelic expression is observed in most patients with sporadic BWS without cytogenetic abnormalities^{16,17}. Loss of imprinting can be associated with *H19* hypermethylation and silencing¹⁸, or apparently with translocation in *KvLQT1*^{19,20}, another closely linked maternally expressed gene. Whether recently described and relatively infrequent mutations of *p57^{Kip2}* in BWS patients²¹ also lead to loss of imprinting or overexpression of *IGF2* remains to be seen. *p57^{Kip2}* mutation in the mouse is associated with some BWS features, such as omphalocele (abdominal wall defect), but not with prenatal overgrowth and multiple organ overgrowth^{22,23}.

It is therefore important to establish whether *IGF2* overexpression is responsible for some or all of the BWS symptoms. Several genetic systems in the mouse lead to increased *Igf2* mRNA or IGFII serum levels, such as mutations in the *H19* (refs 5, 6) and *Igf2r* (refs

24–26) genes, and paternal duplication of distal chromosome 7 (in chimaeras)²⁷. These lead to enhanced fetal growth, with or without other BWS-like phenotypes. However, this is indirect evidence, and a transgenic system in which *Igf2* is itself overexpressed, preferably at different levels, is required to substantiate its involvement in BWS. Previous attempts at producing transgenic mice that overexpress *Igf2* during development were unsuccessful²⁸. An alternative approach was therefore developed in which *Igf2* expression constructs were introduced into embryonic stem (ES) cells. We now show that overexpression of *Igf2* in chimaeras made with transgenic ES cells leads to development of most of the BWS phenotypes in a fashion dependent on *Igf2* mRNA dosage.

Surprisingly, the *Igf2* transgenes were repressed during development and the endogenous *Igf2* locus was hyperactivated. Concomitantly, the transgene lost DNA methylation and the endogenous *Igf2* gene became hypermethylated in a putative silencer sequence. These findings suggest that there is ‘repressor competition’ in imprinted genes, which may be a significant component of the imprinting mechanism.

Establishing *Igf2* transgenic ES cell lines

The construct (pBS *Igf2* mcs-neo) consists of a 16.5-kilobase *EcoRI* fragment containing the *Igf2* locus, including the strong fetal promoters 2 and 3 and a neomycin resistance gene (Fig. 1a). A unique sequence tag was inserted into the 3′ untranslated region to provide a marker for the transgene at both the RNA and DNA levels. This construct was electroporated into HM1 ES cells. The presence of the *Igf2* portion of the transgene in G418-resistant ES cell clones was determined by the polymerase chain reaction (PCR). Of 28 clones analysed, 25 had retained the *Igf2* transgene. The three negative clones, as well as the parental HM1 cells, were used as controls in subsequent experiments. The copy number of the transgene was determined in 10 clones by Southern blotting: each had a single-copy insertion. Reverse transcription (RT)–PCR analysis (with primers A and B) was carried out on the 25 transgene-positive clones, and all were found to express the transgene (Fig. 1b).

The ES cell lines were differentiated *in vitro* (into endoderm) and the expression levels of total *Igf2* transcripts analysed by northern blotting (data not shown). High levels of *Igf2* transcripts were induced and the transgenic cell lines expressed higher levels of *Igf2* mRNA than the control cell lines (1.8- to 3-fold).

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Overexpressing *Igf2* causes fetal overgrowth

Chimaeras were made with five transgenic cell lines (B11, P1, P5 and A15, all with a high level of *Igf2* expression, and B12, with an intermediate level of *Igf2* expression; see Tables 1 and 2) and three control cell lines (A21, B4 and HM1) by microinjection of ES cells into F₂ host blastocysts. Analysis was made of the resulting fetuses or offspring by glucose phosphate isomerase (GPI) isoenzyme analysis (to assess chimaerism), determination of wet weights, and assessment of morphology. A summary of the numbers of chimaeras obtained at fetal and neonatal stages is given in Table 1.

On day 13 of gestation, there was a striking increase in fetal weight with the increasing contribution of transgenic cells (Fig. 2a, d). The relative overgrowth with line B11 was 131% at 40–50% chimaerism. Lines P5 and P1 resulted in a similar extent of overgrowth, whereas with line B12 the effect was more moderate (116% weight at 40–50% chimaerism; Fig. 2d). A regression analysis showed a highly significant effect of chimaerism on weight observed with transgenic cell line B11, but not with control B4 (Fig. 2e). By contrast, on day 12 of gestation there was no apparent effect of chimaerism on fetal weight. The growth response to *Igf2* overexpression therefore arises between days 12 and 13 of development.

Other phenotypes were observed, including an elongated anterior–posterior axis (Fig. 2a), and high-level chimaerism (>50%) with high-level *Igf2*-expressing lines (B11, P1, P5, A15) resulted in fetal lethality from day 13. One of the more obvious phenotypes in high-contribution chimaeras was polyhydramnios, or excess fluid in the amniotic cavity, which is a characteristic BWS phenotype. This was particularly apparent in moribund chimaeras on day 14.

Neonatal overgrowth is disproportionate

At neonatal stages (day 1 after birth), the presence of transgenic ES cells had a strong effect on wet weights of chimaeras derived from lines B12, B11, P1 and P5 (Fig. 2b, c, f). Relative overgrowth at 40–50% chimaerism was 127% with line B12, 146% with B11, 160% with P1, and 161% with P5 (Fig. 2f). With all these cell lines, therefore, the relative increase in size was greater at birth than on day 13 of gestation. Chimaerism was analysed in 15 organs and tissues (by GPI) in chimaeras from the B11 and B12 lines and B4 control; individual tissue contribution was slightly variable, as expected, but there was no evidence of strong positive or negative selection of experimental cells in any tissue (data not shown).

Growth effects on individual organs were also analysed, as there is multiple organ overgrowth in BSW²⁹. In neonatal chimaeras derived from B11, P1 and P5, the heart, kidney, liver and tongue showed a striking and disproportionate increase in size (Fig. 3). For example, with line P1 the neonatal weight increase was to 160% (40–50% chimaerism), but the increase in weight in kidney was to 223% and in heart to 265% (Fig. 3). Histological analysis of kidneys did not reveal any abnormalities; the heart showed a thickened ventricular muscle wall and dilated atria (S. Fleming *et al.*, unpublished data). The liver and tongue were also disproportionately enlarged (to 176 and 175%, respectively, in line B11). The brain, in contrast, did not reveal any increase in weight, and was therefore smaller in relation to body size (100% in line B11).

Some skeletal abnormalities were also observed. Approximately half the chimaeras from lines A15, B11, P1 and P5 had a terminal kink of the tail (Fig. 2b). Furthermore, all high-level chimaeras from lines A15, B11, P1 and P5 died within 24 h of birth. They were lethargic, did not suckle, and seemed to gasp for breath. The lungs were often incompletely aerated, but were otherwise histologically normal. The cause of death is unknown, but may be due to specific organ or endocrine failure. Blood glucose concentrations measured in P1 and B11 chimaeras were in the normal range.

Overgrowth depends on *Igf2* mRNA dosage

The total level of *Igf2* transcripts was determined by northern

blotting in chimaeric fetuses at days 12 and 13, and individual neonatal organs (Fig. 4; summarized in Table 2). In fetuses at day 13, increases of 1.5-fold (B12) and 2.2-fold (B11) were measured at 40–50% chimaerism (Fig. 4a, c). *Igf2* mRNA levels were increased in transgenics in all neonatal organs examined (for example, in kidney the increase was 2.0-fold (for B11) and 2.6-fold (for P1) at 40–50% chimaerism; Fig. 4b, d), with the exception of brain (Fig. 4d). In all cases, the full-length promoter-3 transcript of 3.8 kb was the predominant transcript, and the relative abundance of the various *Igf2* transcripts was not significantly altered. *Igf2* mRNA levels were already elevated in fetal chimaeras at day 12 (B12, 1.4-fold; B11, 2.1-fold; 40–50% chimaerism; Fig. 4a), although at this stage there was no apparent effect on growth. Overall there was an excellent correlation between the increase in transcripts and the increase in weight both at the level of whole embryos at day 13 (compare Fig. 2d with Fig. 4c) and individual neonatal organs (compare Fig. 3c, d with Fig. 4d).

Serum concentrations of IGFII peptide were determined in newborn chimaeras. These were elevated up to 2.7-fold in transgenic chimaeras from B11, up to 2.2-fold from P1, and up to 1.4-fold in B12. Serum IGFII levels in these three groups of chimaeras were significantly different from controls (D. Hill *et al.*, unpublished data).

The *p57^{Kip2}* and *H19* transcripts were also measured in differentiated ES cells and day-13 chimaeras. In differentiated ES cells, *p57^{Kip2}* levels were similar in controls and transgenic cells, whereas

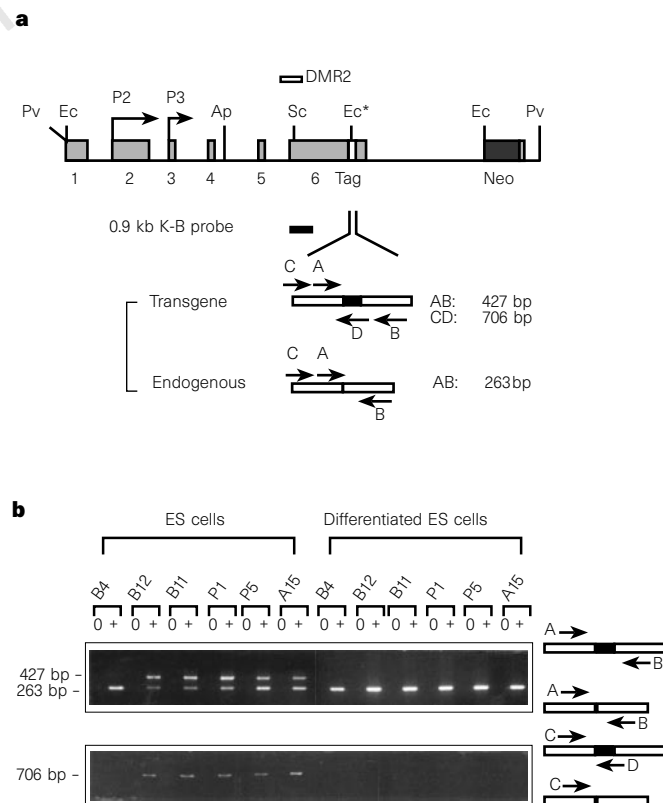


Figure 1 Introduction and expression of *Igf2* transgene construct in ES cells. **a**, The construct pBS-*Igf2* mcs-neo is based on a 16.5-kb *EcoRI* fragment containing the *Igf2* locus from exon 1 to ~6 kb into 3' flanking sequence. The fetal promoters P2 and P3 and the differentially methylated region 2 (DMR2) are indicated. The construct contains a polylinker tag in the 3' untranslated sequence and a selectable marker (Neo). Relevant restriction sites are: Pv, *PvuI*; Ec, *EcoRI*; Sc, *SacII*; Ap, *ApaI*; Ec*, the *EcoRI* site unique to the transgene. The probe used for northern and southern analysis (0.9 kb *KpnI*-*Bam*HI, 0.9 kb K-B) is indicated, as are PCR primers (A–D) used for amplification of DNA and cDNA. **b**, RT-PCR (+ with RT, 0 without RT) was carried out with primers AB (top) or CD (bottom). B4, control cell line; B12–A15, transgenic cell lines.

H19 levels were slightly lower in transgenic cells (mean, 0.74× control; data not shown). The same pattern was observed in day-13 chimaeras: *p57^{Kip2}* was unaffected, but *H19* transcripts were reduced (Fig. 4c).

Transgene repression and *Igf2* hyperactivation

We determined the relative levels of transcription from the transgene and the endogenous gene, the combination of which leads to the overexpression documented above. RT–PCR was performed using primers A and B, which span the 164-base-pair sequence tag inserted into the 3′ untranslated region (UTR) of exon 6 (Fig. 1). In differentiated ES cells, the endogenous transcript was readily amplified, but PCR products corresponding to the transgene were not detectable (Fig. 1b). Because endogenous *Igf2* transcription is strongly induced upon differentiation, competition during PCR could have led to the failure of the transgene product to amplify. RT–PCR was therefore carried out with transgene-specific primer

set C and D on the same cDNA samples. The transgene-specific transcript was detected in undifferentiated ES cells (as shown before) but not after differentiation (Fig. 1b). Transcripts from the transgene were also not detected using this RT–PCR strategy in day-13 chimaeric fetuses and in neonatal organs (not shown, but see Fig. 5).

To confirm this surprising result, RNase protection experiments were performed using an antisense probe for the 3′ UTR overlapping the 3′ end of the transgene tag (Fig. 5). Only trace amounts of transgene-specific protection products were obtained in differentiated ES cells, day-13 chimaeric fetuses, and in neonatal organs (Fig. 5; the only transgene-specific signal reproducibly detected was in line B12, and constituted maximally 1/30 of the level of the endogenous transcript). The combined results show that the *Igf2* transgene becomes repressed during development, and therefore that the endogenous *Igf2* gene becomes hyperactivated and produces the measured increase in transcript levels.

Table 1 Summary of production of chimaeras by blastocyst injection of transgenic ES cells

Cell line	Stage analysed	Blastocysts transferred	Embryos or newborn pups	Embryos or newborns developed from transferred embryos (%)	Chimaeras	Overgrown chimaeras	Contribution of transgenic ES cells in chimaeras (%)		
							0–30 chimaeras	30–60 chimaeras	60–100 chimaeras
HM1(c)	P1	20	14	70	6	0	2	3	1
A21(c)	P1	20	15	75	6	0	2	4	0
B4(c)	E12	30	20	67	18	0	3	5	10
	E13	30	25	83	23	0	8	10	5
B12(i)	P1	20	17	85	8	0	2	4	2
	E12	30	24	80	20	0	4	10	6
B11(h)	E13	40	35	88	23	7	8	11	4
	P1	50	43	86	22	6	10	10	2
B11(h)	E12	32	29	91	23	0	7	8	8
	E13	40	29	73	24	18	4	14	6
P5(h)	P1	60	26	43	18	13	5	13	0
	E12	30	24	80	18	0	5	7	6
P1(h)	E13	30	26	87	13	12	4	3	6
	P1	50	19	38	17	14	6	11	0
A15(h)	P2	30	14	47	7	4	5	2	0

E, day of embryonic development; P, day of postnatal development. A chimaera was designated overgrown when its weight exceeded the mean weight of controls plus three standard deviations; h, high-expressing line; i, intermediate-expressing line; c, control. Chimaerism in newborn offspring was determined in blood and organs and was averaged.

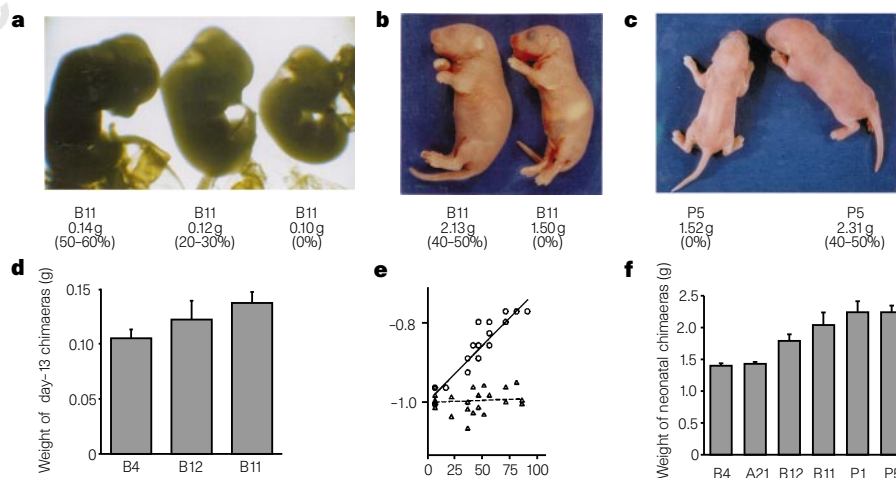


Figure 2 Fetal and postnatal overgrowth in transgenic chimaeras. Chimaeras were made by microinjection of transgenic (B11, B12, P1 and P5) and control (B4 and A21) ES cells into F₂ host blastocysts and analysed on day 13 and the day of birth. **a**, Day-13 B11 chimaeras with their wet weight and percentage chimaerism. Note the elongated anterior–posterior axis of the high-level chimaera (left). **b**, B11 chimaera (left) on the day of birth together with a non-chimaeric littermate (right). Note the kink in the terminal third of the tail in the overgrown chimaeric pup. **c**, Overgrown chimaera (right) of line P5 together with non-chimaeric littermate (left) on the day of birth. **d**, Wet weight of chimaeras on day 13; 40–50% chimaeras were selected for analysis (B4, 0.105 ± 0.008 g, n = 7; B12, 0.122 ± 0.017 g, n = 8; B11, 0.137 ± 0.010 g, n = 9). **e**, Regression analysis of chimaerism (0–100%) on the log₁₀ of fetal weight for B11 transgenic line (circles) and B4 control (triangles). $R_{B11} = 0.94$, $R_{B4} = 0.09$, $P < 0.001$. **f**, Summary of body weights of control (B4 and A21) and experimental (B12, B11, P1 and P5) chimaeras on the day of birth, all at 40–50% chimaerism. Chimaerism was determined in blood and various organs and averaged. Mean ± standard deviation and number of samples are: B4, 1.40 ± 0.038 g, n = 6; A21, 1.43 ± 0.029 g, n = 3; B12, 1.79 ± 0.104 g, n = 4; B11, 2.04 ± 0.197 g, n = 13; P1, 2.24 ± 0.174 g, n = 5; P5, 2.26 ± 0.105 g, n = 4. All experimental groups were significantly different from control groups ($P < 0.001$, Welch test).

Several controls were performed to exclude other explanations. RNase protections were performed with a probe overlapping the 5' end of the sequence tag. Again, no transgene-specific protection fragments were detected (data not shown), excluding the possibility that premature termination occurred within the tag. In addition, combined protection with an exon-4 probe and the 3' UTR probe was performed, and the ratio of the two signals determined. In all cases this ratio was constant, showing that there was no premature termination of a transgenic transcript between exon 4 and the 3' UTR. As an additional control, the transgene construct was transfected into C2 myoblast cells. In differentiated transgenic C2 myoblast cells, high levels of transgene transcripts were detected by both RT-PCR and RNase protection assay (T. Krüger *et al.*, unpublished data). Therefore the transgene has the potential to express high-level, full-length transcripts in differentiated cells, and the RNase protection probes used are capable of detecting them.

DNA methylation

The observation that the transgene construct could be expressed in differentiated cells (C2), but when introduced into ES cells was

repressed during development, suggested an epigenetic mechanism. We therefore determined DNA methylation in the differentially methylated region (DMR)2 (Fig. 1), which is normally methylated on the paternal (expressed) allele of the *Igf2* gene and has been proposed to function as a silencer suppressible by methylation^{8,9}. Methylation was assessed by *Sac*II digestion, and transgenic and endogenous DMR2 were distinguished through the use of an *Eco*RI site present in the transgene tag (Fig. 1). The results show (Fig. 6a, b) that the *Sac*II site in the transgenic DMR2 became methylated *de novo* in undifferentiated ES cells, resulting in DMR2 being highly methylated in both transgene and endogenous gene. This *de novo* methylation did not extend to the CpG island associated with promoter 2 (data not shown). Strikingly, in day-13 fetal chimaeras, the transgenic DMR2 was demethylated, whereas the endogenous DMR2 showed a strong increase in methylation from 40 to ~60% (Fig. 6c, d). These epigenetic changes are consistent with a model in

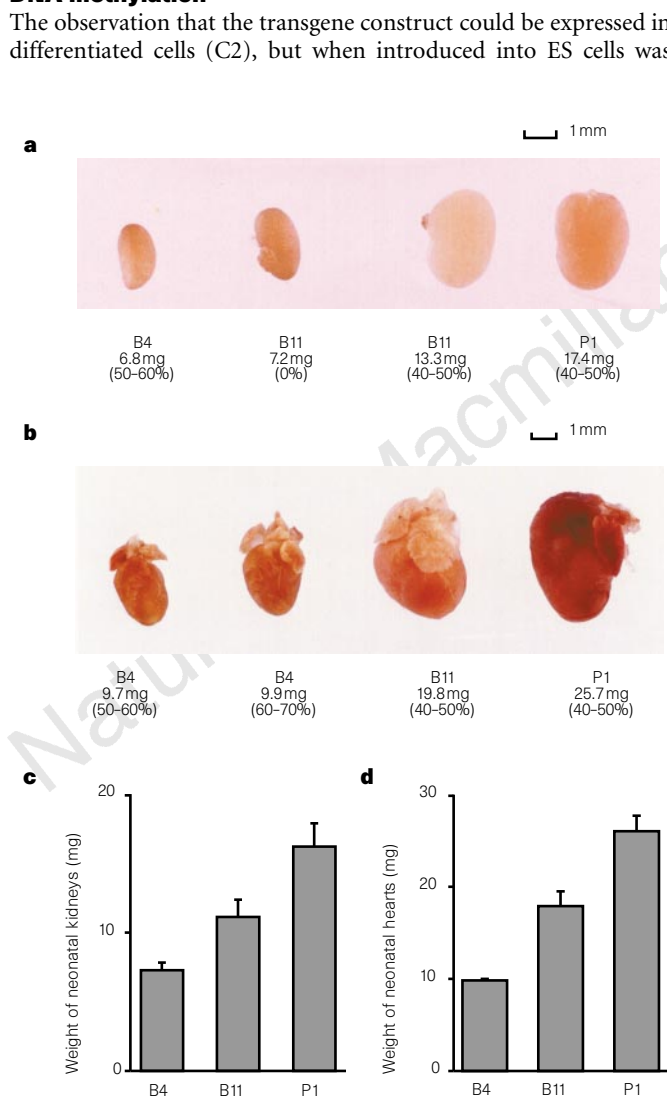


Figure 3 Disproportionate organ overgrowth in transgenic chimaeras. **a, b**, Overgrowth of kidneys (**a**) and hearts (**b**) on the day of birth in transgenic lines B11 and P1 compared with B4 and non-chimaeric controls. **c**, Comparison of weights of neonatal kidneys at 40-50% chimaerism. B4, 7.28 ± 0.55 mg, $n = 6$; B11, 11.12 ± 1.25 mg, $n = 13$; P1, 16.20 ± 1.68 mg, $n = 5$. The experimentals differ for the control ($P < 0.05$, Welch test). **d**, Comparison of weight of neonatal hearts at 40-50% chimaerism. B4, 9.84 ± 0.16 mg, $n = 6$; B11, 17.90 ± 1.6 mg, $n = 13$; P1, 26.03 ± 1.68 mg, $n = 5$. The experimentals differ from the control ($P < 0.05$, Welch test).

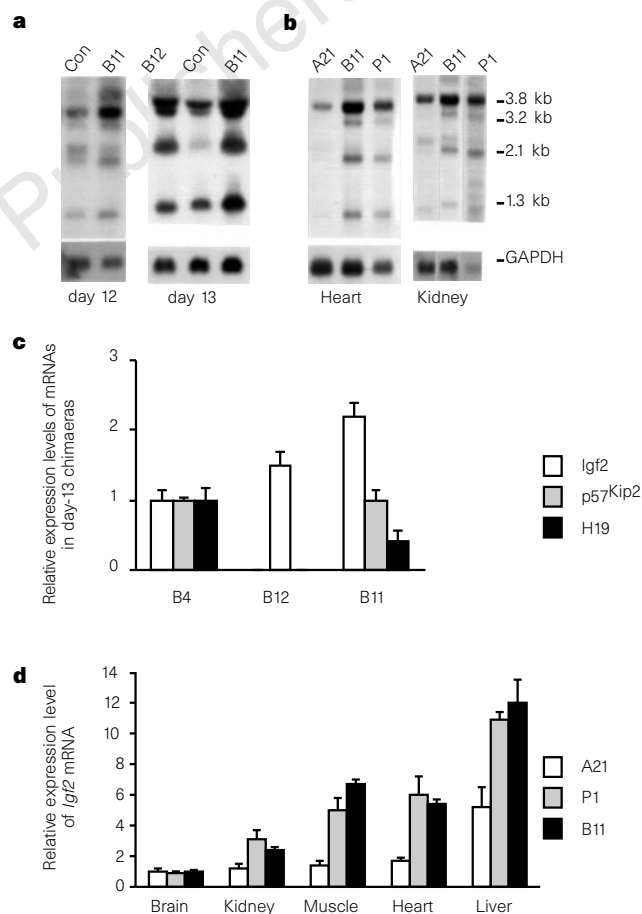


Figure 4 Overexpression of *Igf2* mRNA in transgenic chimaeras. Northern blots of total RNA from fetuses and neonatal organs at days 12 and 13, hybridized with the K-B fragment. The various *Igf2* transcripts have been described⁴⁴. Total levels of *Igf2* transcripts (all transcripts) were quantified against GAPDH control by phosphorimager. **a**, Day-12 chimaera B11 (70-80% chimaeric) and non-chimaeric control (Con) littermate. Day-13 chimaeras B12 (100%) and B11 (20-30%). **b**, Neonatal heart and kidney, in B11 (40-50%), P1 (40-50%) and A21 control chimaeras (20-30%). **c**, Relative expression levels of *Igf2* mRNA in day-13 chimaeras, all at 40-50% chimaerism (mean of controls set arbitrarily at 1.0). B4, 1.0 ± 0.15 , $n = 5$; B12, 1.5 ± 0.19 , $n = 6$; B11, 2.2 ± 0.19 , $n = 8$. Relative expression levels of *p75*^{Kip2} and *H19* are also shown (mean of controls set at 1.0). For *p75*^{Kip2}, B4 (1.0 ± 0.04 , $n = 3$), B11 (1.0 ± 0.14 , $n = 4$); for *H19*, B4 (1.0 ± 0.16 , $n = 3$), B11 (0.41 ± 0.15 , $n = 4$). **d**, Summary of *Igf2* mRNA analysis in neonatal tissues (all at 40-50% chimaerism). Chimaerism was determined in tissues individually. The mean of the control A21 brain is set at 1.0. A21, $n = 4$; P1, $n = 3$; B11, $n = 8$.

which methylation of DMR2 is associated with expression of the *Igf2* gene, and hypermethylation of the endogenous *Igf2* alleles leads to overexpression.

Discussion

We made two major observations. First, overexpression of *Igf2* in mice results in the dosage-dependent appearance of most of the symptoms of the human fetal overgrowth syndrome, BWS. This establishes *Igf2* overexpression as a key determinant of BWS. Second, the surprising findings were made that *Igf2* transgenes

were repressed during development and the endogenous *Igf2* locus was hyperactivated. One possible explanation for these findings is that 'repressor competition' occurs in imprinted genes, which may be a significant component of the imprinting mechanism. As a result of these events, the tissue-specific overexpression of *Igf2* in the transgenics follows that of the endogenous gene and, quite inadvertently perhaps, this may have resulted in a more faithful model of BWS than if the transgene had been expressed.

A mouse model for BWS

The main phenotypes caused by *Igf2* overexpression were pre- and postnatal overgrowth up to 160% at birth, polyhydramnios, fetal and neonatal lethality, disproportionate organ overgrowth including macroglossia, and skeletal abnormalities. Thus, of the main BWS symptoms, only hemihypertrophy, exomphalos, hypoglycaemia and tumours were not observed. Fetal overgrowth was first apparent on day 13 of development, despite the fact that transcript levels were elevated by day 12. A major transition during development occurs between days 12 and 13, when the important events of organogenesis are being completed and exponential fetal growth is initiated. Consistent with this, in BWS, first trimester intrauterine growth is normal and overgrowth is first apparent in the second trimester²⁹. In BWS, birth weights of up to 5.5–6.0 kg have been recorded, which corresponds to the maximum weight increase of 160% seen in our transgenic offspring at birth³⁰.

Overexpression of *Igf2* and organ overgrowth were restricted to those neonatal organs that normally express *Igf2*. Organ overgrowth was disproportionate in those organs showing a considerable increase in *Igf2* transcript levels, in particular kidney, heart, liver and tongue. The direct relationship between levels of *Igf2* overexpression in an organ and its size suggests that IGFII plays an important short-range paracrine/autocrine role in the overgrowth syndrome²⁹. In BWS, disproportionate organomegaly is also observed in organs with the highest level of *IGF2* expression (such as the kidney)²⁹. In addition, there is relative microcephaly, and this was also observed in our mice. However, neither precise levels of *IGF2* transcription nor of IGFII serum levels are known in BWS. Curiously, although in the transgenic pups the tongue was disproportionately overgrown, a protruding tongue, one of the characteristic features of BWS, was never observed. This may be due to the different shape of the skull, and particularly the jaws, in mice.

The fact that we did not observe hemihypertrophy or exomphalos is interesting because these are the only BWS symptoms for which a genotype/phenotype correlation has been found³¹. It has recently been suggested that exomphalos is particularly frequent in those BWS patients with *p57^{Kip2}* mutations, which are quite rare in BWS (~10%) (W. Lam *et al.*, unpublished data). Our chimaeric mice expressed normal levels of *p57^{Kip2}*, which is consistent with the absence of exomphalos. Exomphalos or omphalocele is one of the phenotypes observed in the *p57^{Kip2}* knockout mouse^{22,23}.

Children with BWS have an increased risk of childhood malignancies, in particular Wilms' tumour, but so far no tumours have been observed in the mouse model. This may be because chimaeras expressing high levels of *Igf2* die, or because there was a low incidence of tumours. The histology of the disproportionately enlarged kidneys was normal at birth (S. Fleming *et al.*, unpublished data), but further work is required to see whether premalignant lesions characteristic of BWS are observed at later stages.

The *Igf2* dosage-dependent phenotypes seen in this transgenic system therefore provide an excellent model of BWS, and overlap with other fetal overgrowth syndromes attributed to increased IGFII levels (such as the 'IGFII overgrowth syndrome'³², in which symptoms are less severe, and the Simpson–Golabi–Behmel syndrome³³, in which symptoms are more severe than in BWS). This is a better model than either the *H19*-mouse (overgrowth only) or the *Igf2r*-mouse (disproportionate overgrowth of the heart but not other organs, and an overall increase in birth weight to 130%). Exomphalos

Table 2 Expression of *Igf2* mRNA in transgenic ES cells, chimaeras and their phenotypes

Cell line	Expression of <i>Igf2</i> transgene in ES cells*	Expression level of <i>Igf2</i> mRNA in fetuses and neonates†	Fetal and neonatal overgrowth‡	Fetal and neonatal lethality
Controls	HM1	–	+	–
	A21	–	+	–
	B4	–	+	–
Transgenic ES cell lines	B12	+	++	–
	A15	+	+++	+
	P1	+	+++	+
	B11	+	+++	+
	P5	+	+++	+

* Determined by RT-PCR (Fig. 1).

† Determined by quantitative northern blots in day-13 fetuses and neonatal tissues (Fig. 4).

‡ Level of *Igf2* mRNA transcripts in controls (1.0); ++, elevated level up to 2.0-fold; +++, elevated level between 2.0- and 3.0-fold.

§ See Table 1 for details. +, up to 130% birth weight as compared to controls; ++, more than 130% birth weight.

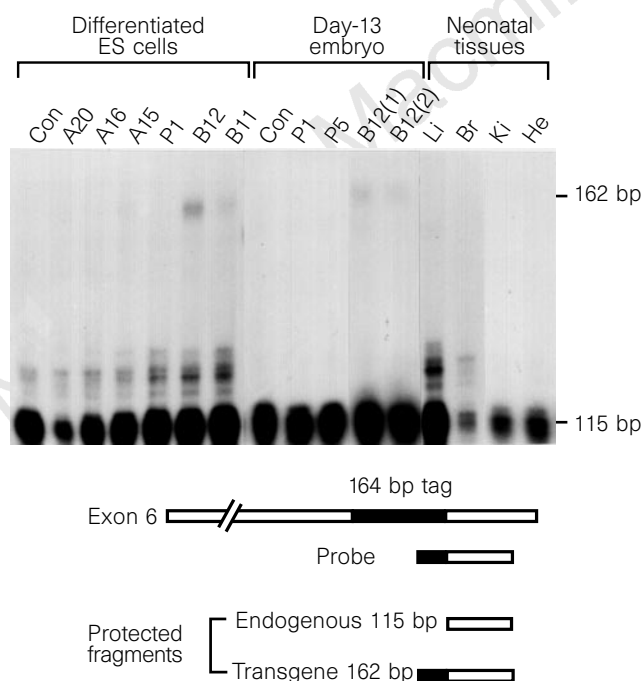


Figure 5 RNase protection assays to distinguish transgenic and endogenous *Igf2* transcripts. The RNA antisense probe contains 47 bp of the transgene-specific tag and 115 bp of *Igf2* exon 6 sequence. Transgene mRNA protects a 162-bp fragment, and full-length endogenous *Igf2* mRNA protects a 115-bp fragment. RNA from differentiated ES cells (Con is A21), day-13 chimaeras (Con is B4, 40–50% chimaerism; P1, 50–60%; P5, 60–70%; B12, 90–100%), and neonatal tissues from a B11 chimaera (40–50%) was analysed. Note that only trace amounts of the transgene RNA (162 bp) were detected. <1/30 of the endogenous RNA as determined by phosphorimager. The relative levels of endogenous *Igf2* mRNA between samples cannot be compared as loading was not controlled in this experiment.

or omphalocele, enlargement of the adrenal cortex, and renal medullary dysplasia have been observed in the $p57^{Kip2}$ knockout mouse^{22,23}, together with some other phenotypes that might constitute minor features of BWS. Therefore, overexpression of *Igf2* and underexpression of $p57^{Kip2}$ seem to account for virtually all of the symptoms of BWS, with the possible exception of tumours and hypoglycaemia, of which the latter could be a result of altered imprinting/expression of the gene encoding insulin.

'Repressor competition'

The *Igf2* transgene was expressed in ES cells at a level similar to the endogenous gene, and became methylated *de novo* in DMR2. However, during development the transgene was repressed, presumably because it lacks the *H19* enhancers or other *cis*-linked enhancers⁴, and the endogenous *Igf2* gene was hyperactivated. Concomitantly, methylation was lost from transgenic DMR2, and gained by endogenous DMR2. Because the endogenous *Igf2* gene became methylated to more than 50%, the maternal allele must have become methylated in some cells. Whether the maternal allele is now expressed (that is, there is loss of imprinting) or whether the paternal allele is hyperactivated cannot be assessed in our genetic system. The observation that *H19* was downregulated also suggests that the maternal copy of *Igf2* is expressed, which could lead,

through enhancer competition, to downregulation of the maternal copy of *H19*. Indeed, a substantial number of BWS patients with biallelic *IGF2* expression show downregulation of *H19* (ref. 17). It is remarkable that the upregulation of endogenous *Igf2* mRNA could be very substantial, four- to fivefold in transgenic cells of a 50% chimaera which showed a two- to threefold overall increase. However, four- to fivefold overexpression of *Igf2* mRNA was also observed in the paternal disomy of distal chromosome 7, showing that two active copies of *Igf2* have the potential to produce more than a twofold increase in mRNA³⁴. Whether this regulation is at the transcriptional or post-transcriptional level is not known.

One model that could account for these observations is 'repressor competition'. DMR2 or perhaps other regions in the transgene could bind *trans*-acting repressor molecules following differentiation of ES cells. These would preferentially, but perhaps not exclusively, bind to the non-methylated (maternal) copy of DMR2 and would be dosage-sensitive (limiting). Because the transgene represents additional targets for limiting repressor molecules, binding of repressor to either endogenous *Igf2* allele is reduced. The increase in methylation in the endogenous DMR may be a consequence of lack of repressor binding in this region, as DMR2 seems to have a propensity to become methylated *de novo*. It is likely that transactivation is specific for the *Igf2* gene, in particular because of the tight dose-response relationship of *Igf2* mRNA level and growth phenotype, but evidence for this will rely on genetic experiments.

An alternative possibility is that upregulation of the endogenous *Igf2* gene is a consequence of transient expression of the *Igf2* transgene. The excess IGFII expressed from the transgene in undifferentiated cells in the early embryo could result in hyperactivation of one or both endogenous alleles, possibly through a positive feedback mechanism. This could be accompanied by changes in methylation of the endogenous alleles that persist in the developing embryo, thereby leading to sustained overexpression.

There is evidence from other studies that dosage-sensitive mechanisms may be involved in the control of imprinting. First, some kind of chromosome counting has been implicated in the control of allelic methylation in some imprinted genes³⁵. Second, a recent study shows that a *U2af1-rs1* transgene leads to hypermethylation of the imprinted endogenous *U2af1-rs1* gene³⁶. Third, the imprinted *Xist* gene as a transgene can lead to reactivation of the otherwise silent endogenous copy of *Xist*^{37,38}. Therefore, imprinting and counting of copies of genes may be mechanistically linked and involve dosage-sensitive *trans*-acting molecules.

If indeed a repressor (or repressors) exists that explains our observations, mutations in this repressor could lead to increased *Igf2* expression and the symptoms of BWS. As noted previously, mutations in BWS seem to be heterogenous ($p57^{Kip2}$, *KvLQT*, *H19* methylation), and it is therefore possible that one of these genes, or another as-yet unidentified BWS gene, encodes a repressor of the type proposed from these experiments. □

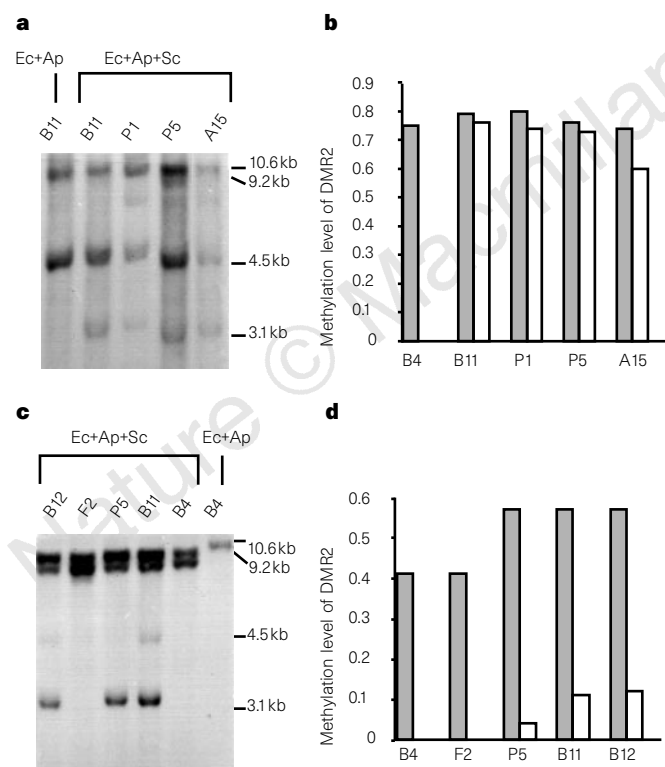


Figure 6 Methylation of DMR2 in transgenic and endogenous *Igf2*. DNA was restricted by *EcoRI* and *ApaI*, with or without *SacII*, to reveal methylation of the *SacII* site in DMR2 of the transgene (4.5 kb methylated fragment, 3.1 kb unmethylated) and endogenous *Igf2* gene (10.6 kb methylated, 9.2 kb unmethylated). Hybridization was with the K-B fragment. **a**, Undifferentiated transgenic ES cell lines. **b**, Methylation levels in undifferentiated cells as determined by phosphorimager scanning (methylation index = methylated/methylated + unmethylated). Black columns represent the endogenous alleles; white columns represent the transgene. The index includes a correction for the reduced intensity of the 9.2-kb and 3.1-kb bands, as the *SacII* site is within the probe. **c**, Day-13 transgenic and control chimaeras. B4, (80–90% chimaerism) and F2 (non-chimaeric) are controls; B12 (90–100% chimaerism), P5 (90–100%) and B11 (90–100%) are transgenic chimaeras. **d**, Phosphorimager quantification of the methylation indices.

Methods

***Igf2* expression construct, electroporation into ES cells, and preparation of chimaeras.** The 16.5-kilobase *EcoRI* fragment containing fetal promoters 2 and 3, the rest of the *Igf2* gene and approximately 6 kb of 3' flanking sequence (for full genomic DNA sequence, see ref. 39; GenBank accession no. U71085) was subcloned from *Igf2* cos 4 (ref. 40) into pBluescript (IKS(+)) (Stratagene)). The 164-base-pair tag comprised the pBluescript II polylinker internally deleted from the *ClaI* to *XhoI* sites and cloned as a *BssHII* fragment into the *MluI* site in the 3' UTR in exon 6 of *Igf2* (resulting plasmid called pBS-*Igf2* mcs). The HSV-TK promoter neomycin gene transcription unit was inserted into the unique *ClaI* site of pBS-*Igf2* mcs to result in pBS-*Igf2* mcs-neo. All cloning procedures followed standard methods⁴¹. The construct was linearized with *PvuI*, which also removes part of the vector sequences, and ~20 µg DNA was electroporated into $1-5 \times 10^7$ HM1 cells at passage 10–12 (a gift from D. Melton), which were maintained on STO feeders⁴². Clones were selected in

G418 ($180 \mu\text{g ml}^{-1}$). *In vitro* differentiation of ES cells into endoderm was performed as described⁴². ES cells from characterized lines at passages 16–26 were microinjected into F₂ (C57BL/6 $\times$ CBA/Ca) $\times$ (C57BL/6 $\times$ CBA/Ca) blastocysts⁴² and transferred into F₁ foster mothers. Chimaerism was analysed by GPI isoenzyme electrophoresis (the ES cells are GPI-aa and the host GPI-bb).

DNA and RNA analysis. DNA and RNA were extracted from cells or tissues according to standard procedures⁴¹, and poly(A)⁺ RNA was prepared with a Promega kit. For RT-PCR, 1 μg of poly(A)⁺ RNA was reverse-transcribed using reverse transcriptase (Promega) and random primers (Promega), according to the manufacturers's instructions. Negative controls (without reverse transcriptase) were always run in parallel. PCR on DNA or cDNA was performed in 50- μl volumes using Boehringer Mannheim reagents. We ran 30 cycles of PCR with denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The primers were: A, 5'-GCATTTCTCCCCATC ATAGG-3'; B, 5'-CAAGCTTTGATTTTGCCAGA-3'; C, 5'-CAAGGTGGGG TGAGGGAG-3'; D, 5'-GCTTTTGTTCCTTTAGT-GAGG-3'. PCR for primers C and D included 5% DMSO. PCR products were electrophoresed on 2% agarose gels. DNA samples (15 μg) were restricted using the manufacturers' conditions (Boehringer; NEB; Promega) and were electrophoresed through 0.8% agarose gels, alkaline-blotted, and hybridized in Church buffer⁸. Probes were random-labelled with [α -³²P]dCTP using the Pharmacia kit. Total RNA (5–10 μg) was electrophoresed through formaldehyde gels, blotted in 10 $\times$ SSC, and hybridized as above. A 427-bp fragment comprising the 164-bp tag (modified pBluescript II polylinker) and 148 bp and 115 bp of *Igf2* sequence flanking the tag 5' and 3', respectively, was amplified from pBS-*Igf2* mcs and cloned in either orientation into pCRII (Invitrogen), to produce plasmids pCR-*Igf2* Rmcs and pCR-*Igf2* Fmcs. An antisense riboprobe running from the 3' flanking sequence into the tag was prepared from pCR-*Igf2* Rmcs by digestion with *Xba*I (*Xba*I site in the pBluescript II polylinker in the tag) and transcription *in vitro* with SP6 RNA polymerase. An antisense probe starting in the tag and extending into the 5' sequence was prepared by digestion of pCR-*Igf2* Fmcs with *Nsi*I and transcription with T7 RNA polymerase (using the T7 promoter in the pBluescript II polylinker). For the probe for exon 4, a *Kpn*I-*Hinc*II fragment was cloned into pBS(+), and the plasmid linearized with *Taq*I for transcription with T3 RNA polymerase. Riboprobes were synthesized with [α -³²P]UTP using enzymes from Stratagene and Promega, according to the manufacturers' instructions. RNase protections were performed according to ref. 43: 100,000 d.p.m. of each probe was hybridized to ~20 μg total RNA.

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The capsaicin receptor: a heat-activated ion channel in the pain pathway

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Capsaicin, the main pungent ingredient in 'hot' chilli peppers, elicits a sensation of burning pain by selectively activating sensory neurons that convey information about noxious stimuli to the central nervous system. We have used an expression cloning strategy based on calcium influx to isolate a functional cDNA encoding a capsaicin receptor from sensory neurons. This receptor is a non-selective cation channel that is structurally related to members of the TRP family of ion channels. The cloned capsaicin receptor is also activated by increases in temperature in the noxious range, suggesting that it functions as a transducer of painful thermal stimuli *in vivo*.

Pain is initiated when the peripheral terminals of a subgroup of sensory neurons are activated by noxious chemical, mechanical or thermal stimuli. These neurons, called nociceptors, transmit information regarding tissue damage to pain-processing centres in the spinal cord and brain¹. Nociceptors are characterized, in part, by their sensitivity to capsaicin, a natural product of capsicum peppers that is the active ingredient of many 'hot' and spicy foods. In mammals, exposure of nociceptor terminals to capsaicin leads initially to excitation of the neuron and the consequent perception of pain and local release of inflammatory mediators. With prolonged exposure, nociceptor terminals become insensitive to capsaicin, as well as to other noxious stimuli². This latter phenomenon of nociceptor desensitization underlies the seemingly paradoxical use of capsaicin as an analgesic agent in the treatment of painful disorders ranging from viral and diabetic neuropathies to rheumatoid arthritis^{3,4}. Some of this decreased sensitivity to noxious stimuli may result from reversible changes in the nociceptor, but the long-term loss of responsiveness can be explained by death of the nociceptor or destruction of its peripheral terminals following exposure to capsaicin^{2,5}.

The cellular specificity of capsaicin action and its ability to evoke the sensation of burning pain have led to speculation that the target of capsaicin action plays an important physiological role in the detection of painful stimuli. Indeed, capsaicin may elicit the perception of pain by mimicking the actions of a physiological stimulus or an endogenous ligand produced during tissue injury⁶. Although the excitatory and neurotoxic properties of capsaicin have been used extensively to define and study nociceptive neurons, its precise mechanism of action has remained elusive. Electrophysiological^{7,8} and biochemical⁹ studies have shown that capsaicin excites nociceptors by increasing the permeability of the plasma membrane to cations, but the molecular mechanism underlying this phenomenon is unclear. Proposed models range from the direct perturbation of membrane lipids by hydrophobic capsaicin molecules¹⁰ to the activation of a specific receptor on or within sensory neurons⁶. Because capsaicin derivatives show structure–function relationships and evoke responses in a dose-dependent manner^{11,12}, the existence of a receptor site represents the most likely mechanism. This model has been strengthened by the development of capsazepine, a competitive capsaicin antagonist¹³, and by the discovery of resiniferatoxin, an extremely potent capsaicin analogue from *Euphorbia* plants that mimics the cellular actions of capsaicin^{14,15}. The potency of resiniferatoxin at nanomolar quantities

has led to its use as a high-affinity radioligand to visualize saturable, capsaicin- and capsazepine-sensitive binding sites on nociceptors¹⁶.

A more detailed understanding of the molecular nature of capsaicin action and its relationship to endogenous pain signalling mechanisms might be obtained through the cloning of a gene encoding a capsaicin receptor. To achieve this we used a functional screening assay to isolate a cDNA clone that reconstitutes capsaicin responsiveness in non-neuronal cells. The deduced amino-acid sequence of this clone demonstrates that the capsaicin receptor is an integral membrane protein with homology to a family of putative store-operated calcium channels. The cloned receptor seems to be

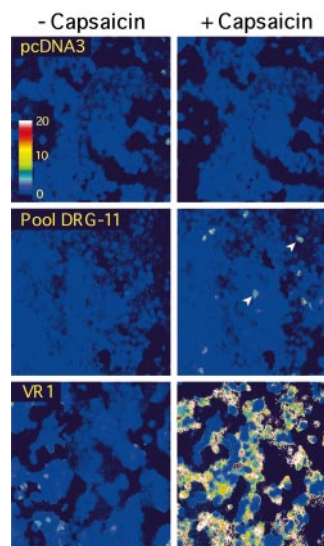


Figure 1 Expression cloning of a capsaicin receptor using calcium imaging. HEK293 cells transiently transfected with pools of clones from a rodent dorsal root ganglion (DRG) cDNA library were subjected to microscopic fluorescent calcium imaging before (left) and during (right) treatment with 3 μ M capsaicin. Cells transfected with vector alone (pcDNA3; top) exhibited no response to capsaicin. Between 1% and 5% of cells transfected with pool 11 exhibited marked increases in cytoplasmic calcium (middle, arrowheads). This pool was iteratively subdivided and reassayed until a single positive clone (VR1) was isolated (bottom). Elevated relative calcium concentrations are indicated by an increased ratio of Fura-2 emission at 340 versus 380 nm wavelength excitation (see colour bar).

expressed exclusively by small-diameter neurons within sensory ganglia, providing a definitive molecular explanation for the remarkable selectivity of capsaicin action. This receptor is also a thermal sensor that is strongly activated when ambient temperatures are elevated to a range known to elicit pain in humans or pain-associated behaviours in animals. Thus capsaicin elicits burning sensations through the activation of a heat-gated ion channel that is likely to contribute to the detection of painful thermal stimuli *in vivo*.

Expression cloning of receptor cDNA

The lack of specific information regarding the molecular structure of capsaicin receptors prompted us to adopt a functional screening strategy for isolating candidate cDNA clones. A mammalian cell expression cloning strategy was devised on the basis of the ability of capsaicin to trigger robust calcium influx into sensory neurons *in vitro*^{9,17}. We reasoned that a capsaicin receptor-encoding cDNA might confer upon non-neuronal cells a similar ability to undergo increases in intracellular free calcium upon exposure to capsaicin, assuming that capsaicin acts at a proteinaceous site and that a single cDNA can confer sensitivity to capsaicin in a heterologous context. Because capsaicin responsiveness seems to be confined to nociceptive neurons with cell bodies that reside within sensory ganglia⁵, a cDNA library was constructed from dorsal root ganglion-derived messenger RNA. This library was subdivided into pools of approximately 16,000 clones, and each pool was transiently transfected into human embryonic kidney-derived HEK293 cells. Transfected cells were then loaded with the fluorescent calcium-sensitive dye Fura-2

(ref. 18), and microscopically examined for capsaicin-evoked changes in intracellular calcium levels. A positive pool was identified (Fig. 1, middle) and iteratively subdivided and reassayed. In this way, an individual clone containing a 3-kilobase (kb) cDNA insert was obtained that, by itself, conferred capsaicin (Fig. 1, bottom) or resiniferatoxin (not shown) sensitivity to transfected HEK293 cells. Because a vanilloid moiety constitutes an essential chemical component of capsaicin and resiniferatoxin structures, the proposed site of action of these compounds is more generally referred to as the vanilloid receptor¹⁶. Accordingly, we have named the newly cloned cDNA VR1, for vanilloid receptor subtype 1.

VR1 and vanilloid receptor pharmacology

To compare the pharmacological properties of the cloned receptor to those of native vanilloid sites in sensory ganglia, we expressed VR1 in *Xenopus* oocytes and used whole-cell voltage-clamp analysis to quantitatively examine the electrophysiological responses to a variety of vanilloid agonists and antagonists. At negative holding potentials, exposure to capsaicin or resiniferatoxin produced dose-dependent inward current responses in VR1-expressing oocytes, but not in water-injected control cells (Fig. 2a). As observed in sensory neurons^{19,20}, capsaicin-evoked current responses returned rapidly to baseline after agonist removal, whereas resiniferatoxin responses often failed to recover, even after a prolonged washout period. Half-maximal effective concentrations for these agonists were within an order of magnitude of those reported for native vanilloid receptors^{8,13}, with resiniferatoxin being approximately 20-fold more potent than capsaicin ($EC_{50} = 39.1$ nM and 711.9 nM,

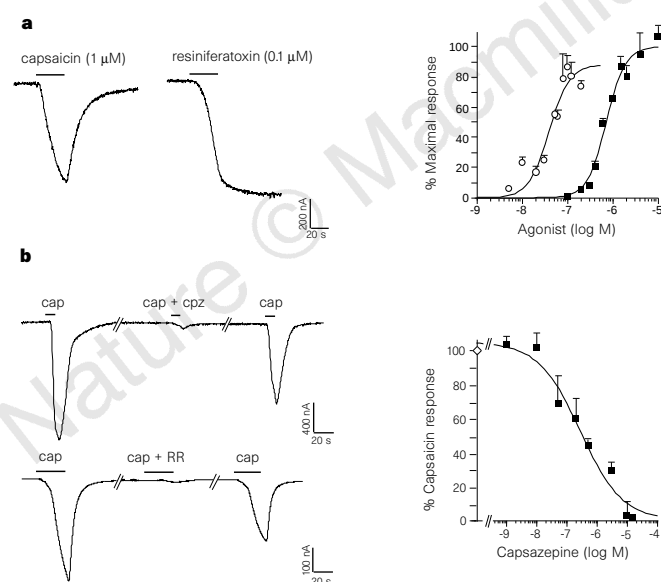


Figure 2 VR1 responds to purified vanilloids and pepper extracts. **a**, Activation of VR1 by capsaicin and resiniferatoxin. Left, agonists were applied sequentially to the same *Xenopus* oocyte expressing VR1. Membrane currents were recorded in the whole-cell voltage-clamp configuration. Bars denote duration of agonist application. Right, concentration-response curve for capsaicin (filled squares) and resiniferatoxin (open circles). Membrane currents were normalized in each oocyte to a response obtained with 1 μ M capsaicin and expressed as a percent of maximal response to capsaicin. Each point represents mean values ($\pm$ s.e.m.) from five independent oocytes. The Hill equation was used to fit the response data. **b**, Antagonism by capsazepine (cpz) and ruthenium red (RR). Current tracing at top left shows reversible block of capsaicin (cap; 0.6 μ M) response by capsazepine (cpz; 10 μ M) after 2 min pretreatment. Slash marks represent washout periods of 2 and 3 min, respectively ($n = 3$). A capsazepine inhibition

curve is shown to the right ($n = 4$ independent oocytes for each point). Current responses were normalized to that elicited by capsaicin alone in each oocyte. (0.6 μ M, open diamond). Current tracing at bottom left shows reversible block of a capsaicin (0.6 μ M)-evoked response by ruthenium red (RR; 10 μ M). Slash marks denote washout periods of 2 and 12 min, respectively ($n = 3$). **c**, Responses to capsaicin (10 μ M) and extracts derived from four varieties of peppers in oocytes expressing VR1 (30 s application). Bottom right, relative potencies of each pepper extract are plotted (mean $\pm$ s.e.m., $n = 3$). Values were normalized in each cell to responses obtained with capsaicin (10 μ M). Extracts evoked no responses in water-injected cells. Reported pungencies for pepper varieties (in Scoville units) are: Habanero (H), 100,000–300,000; Thai green (T), 50,000–100,000; wax (W), 5,000–10,000; and Poblano verde (P), 1,000–1,500 (ref. 23). Capsaicin (C) is rated as 16×10^6 units.

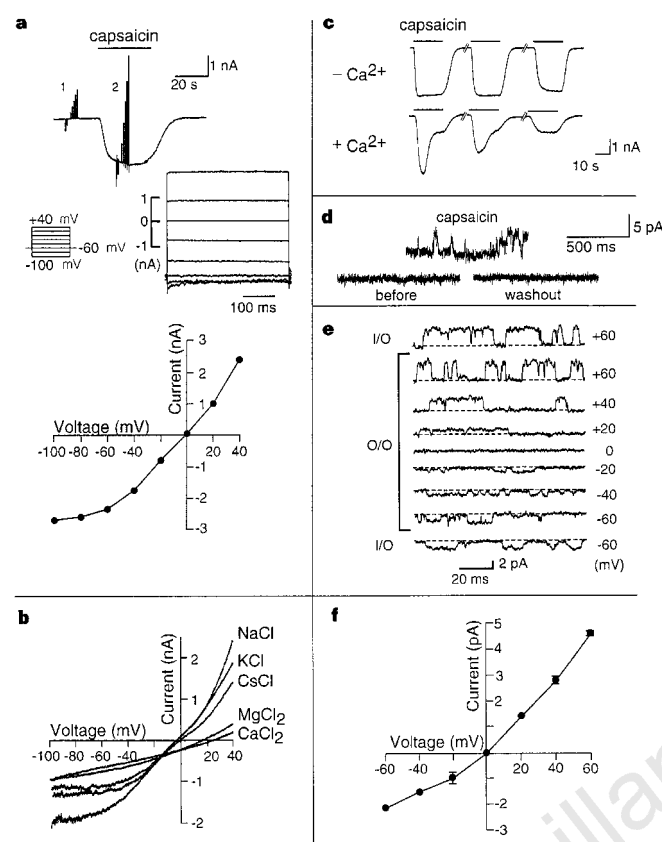


Figure 3 VR1 is a calcium-permeable, non-selective cation channel. Electrophysiological properties of capsaicin-activated currents in VR1-transfected mammalian HEK293 cells. **a**, VR1 currents are time independent, outwardly rectifying, and cation specific. A capsaicin-evoked inward current response (top) was analysed using a series of 400-ms step pulses (–100 to +40 mV; middle, left). Baseline currents (denoted as 1 on top trace) were subtracted from responses in the presence of agonist (2) to yield a series of agonist-evoked responses at different holding potentials (middle, right). These responses show outward rectification when plotted as a function of membrane voltage (bottom). Calcium-free standard bath solution and a caesium aspartate-filled recording electrode were used. **b**, Capsaicin elicits non-selective cation currents in VR1-transfected cells. Voltage ramps (–100 to +40 mV in 500 ms) were used to generate current-voltage curves in bath solutions with the indicated cationic compositions. Recording electrodes were filled with NaCl. Similar results were obtained with KCl- or CsCl-filled electrodes. Replacement of extracellular NaCl (140 mM) with equimolar KCl or CsCl did not significantly shift reversal potential ($E_{rev} = -0.7 \pm 1.2$ mV, $n = 8$; -1.5 ± 1.0 mV, $n = 9$; -4.3 ± 0.9 mV, $n = 8$, respectively; $P_{K}/P_{Na} = 0.94$; $P_{Ca}/P_{Na} = 0.85$). Replacement of extracellular NaCl with isotonic (112 mM) $MgCl_2$ or $CaCl_2$ shifted E_{rev} to 14.4 ± 0.7 mV ($n = 5$) or 24.3 ± 2.3 mV ($n = 7$), respectively ($P_{Mg}/P_{Na} = 4.99$; $P_{Ca}/P_{Na} = 9.60$). **c**, Whole-cell current responses evoked by repeated capsaicin applications show desensitization in calcium-containing standard bath solution, but not in calcium-free solution. Capsaicin ($1 \mu M$) was applied every 5 min and CsCl was used as pipette solution. The ratios of current size at the end of the third application to the peak of the first application were $95.3 \pm 2.6\%$ ($n = 3$) in calcium-free solution, and $13.0 \pm 4.3\%$ ($n = 5$) in calcium-containing solution (t -test; $P < 0.00001$). **d–f**, Single-channel properties of capsaicin-evoked responses. Inside-out (I/O) or outside-out (O/O) patches were excised from VR1-transfected cells and analysed in symmetrical 140 mM NaCl. **d**, Traces obtained from a single O/O patch before, during and after capsaicin ($1 \mu M$) application to the bath solution ($V_{hold} = +40$ mV). Note multiple simultaneous channel openings in the presence of capsaicin. **e**, Traces obtained in the presence of capsaicin at the indicated holding potentials. Broken lines indicate the closed-channel level. No agonist-evoked channel activity was seen in cells transfected with vector alone ($n = 8$, not shown). **f**, Current-voltage curve of mean single-channel amplitudes ($\pm$ s.e.m.) calculated from data shown in **e**, also exhibits pronounced outward rectification.

respectively). Hill coefficients derived from these analyses (1.95 and 2.08, respectively) suggest that full activation of the receptor involves the binding of more than one agonist molecule, again consistent with previously described properties of native vanilloid receptors^{8,16}. Capsaicin-evoked responses in VR1-expressing oocytes were reversibly blocked by the competitive vanilloid receptor antagonist capsazepine at concentrations ($IC_{50} = 283.5$ nM) that inhibit native receptors¹³ (Fig. 2b). Another pharmacological characteristic of vanilloid receptors, is their sensitivity to the non-competitive antagonist ruthenium red¹⁷, which blocked capsaicin-evoked responses in a reversible manner (Fig. 2b). Responses to resiniferatoxin (50 nM) were also reversibly antagonized by capsazepine (5 μM) or ruthenium red (10 μM) (not shown).

As has been recognized for years, the relative pungencies of pepper varieties span an enormously wide range, reflecting, in part, differences in vanilloid content. Methods for rating peppers with respect to their relative 'hotness' have hitherto relied on subjective psychophysical assays²¹ or on the biochemical determination of capsaicin content²². To further explore the connection between the biology and biochemistry of vanilloid action, we sought to determine whether the cloned vanilloid receptor could respond electrophysiologically to pepper extracts in proportion to their ability to evoke pain. Ethanol extracts were prepared from several capsicum varieties and their potencies relative to a saturating dose of capsaicin (10 μM) were determined in the oocyte expression system (Fig. 2c). Indeed, we found that the different 'hotness' of these pepper variants, as determined by subjective psychophysical ratings²³, correlated with their rank order potencies as activators of VR1.

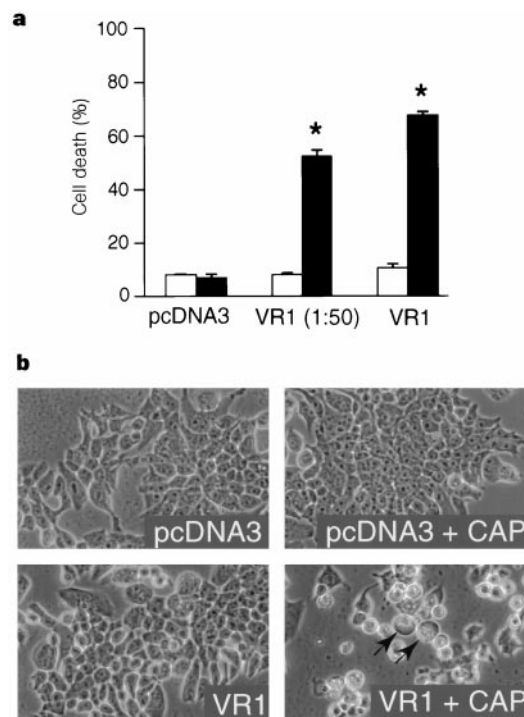


Figure 4 Capsaicin induces death of cells expressing the vanilloid receptor. **a**, HEK293 cells were transiently transfected with either vector alone (pcDNA3), VR1 cDNA diluted 1:50 in pcDNA3, or VR1 cDNA alone. After 7 h at 37°C in the presence of capsaicin (3 μM , black bars) or vehicle (0.3% ethanol, white bars), the percentage of dead cells was determined. Data represent mean $\pm$ s.e.m. of triplicate determinations from a representative experiment. Asterisks indicate a significant difference from ethanol-treated cells (t -test, $P < 0.0001$). **b**, Phase-contrast photomicrographs of parallel cultures transfected with pcDNA3 or VR1 (1:50) before (left) or 4 h after (right, +CAP) addition of capsaicin (3 μM). Note the cytoplasmic swelling and eccentric position of cytoplasmic contents (arrows).

To explore the possibility that capsaicin mimics the action of a known chemical modulator of nociceptor function, we tested agents known to activate sensory neurons for their ability to evoke responses in HEK293 cells or oocytes expressing VR1. None of the agents tested gave positive responses, including adenosine triphosphate (50 μ M), serotonin (10 μ M), acetylcholine (300 μ M), bradykinin (1 μ M), substance P (10 μ M), histamine (10 μ M), glutamate (100 μ M), and hypertonic saline (600 mOsm).

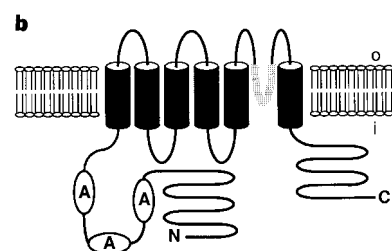
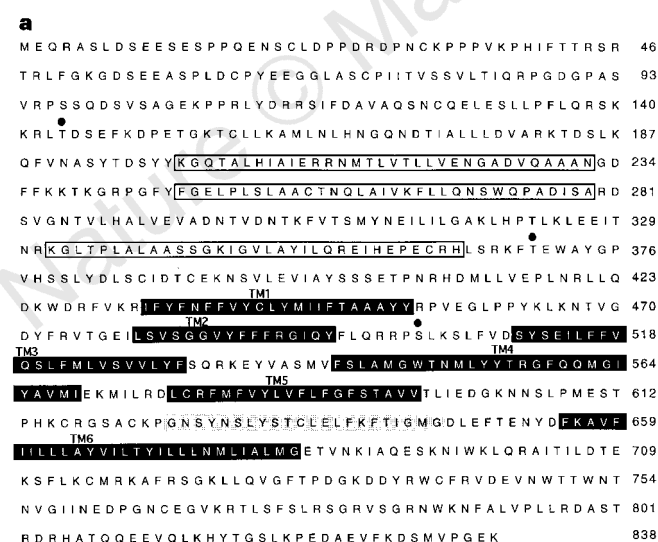
VR1 ion channel has high Ca^{2+} permeability

To characterize more fully the electrophysiological properties of the cloned receptor at both whole-cell and single-channel levels, we performed a series of patch-clamp studies on transfected mammalian cells expressing VR1. In the whole-cell configuration, VR1-transfected HEK293 cells showed robust inward current responses (at a holding potential of -60 mV) that developed with a short latency upon bath application of capsaicin (Fig. 3a). No such currents were observed in cells transfected with vector alone (not shown). In calcium-free medium, the capsaicin-evoked current did not vary with time, either at a constant holding potential of -60 mV or during voltage steps from -100 to $+40$ mV (in increments of 20 mV) (Fig. 3a). This property enabled us to characterize capsaicin-mediated currents under steady-state response conditions in subsequent experiments. Current-voltage relations derived from these data show that such responses exhibit prominent outward rectification resembling that observed in cultured dorsal root ganglion neurons⁸ (Fig. 3a, bottom). Because the observed reversal potential was close to 0 mV ($E_{\text{rev}} = 0.5 \pm 0.9$ mV, $n = 13$), the capsaicin-mediated response must involve the opening of a cation-selective channel. In sensory neurons, vanilloid-evoked currents are carried by a mixture of monovalent and divalent cations⁷⁻⁹, and we therefore conducted a series of ion substitution experiments to examine the relative contributions of various cations to capsaicin-evoked

currents in VR1-expressing cells. Current-voltage relations established for cells bathed in solutions of differing cationic compositions show that VR1 does not discriminate among monovalent cations, but exhibits a notable preference for divalent cations (permeability sequence: $\text{Ca}^{2+} > \text{Mg}^{2+} > \text{Na}^+ \approx \text{K}^+ \approx \text{Cs}^+$) (Fig. 3b). The very high relative permeability of VR1 to calcium ions ($P_{\text{Ca}}/P_{\text{Na}} = 9.60$; $P_{\text{Mg}}/P_{\text{Na}} = 4.99$) exceeds that observed for most non-selective cation channels, and is similar to values reported for NMDA-type glutamate receptors and $\alpha 7$ nicotinic acetylcholine receptors ($P_{\text{Ca}}/P_{\text{Na}} = 10.6$ and 20, respectively)^{24,25}, both of which are noted for this property. With all bath solutions examined, an outwardly rectifying current-voltage relation was observed, although this feature was less prominent in bath solutions containing MgCl_2 or CaCl_2 .

In cultured sensory neurons, electrophysiological analyses of vanilloid-evoked responses have shown them to be kinetically complex and to desensitize with continuous vanilloid exposure^{20,26}. This electrophysiological desensitization (which might underlie aspects of physiological desensitization produced by vanilloids *in vivo*) seems to depend, in part, on the presence of extracellular calcium^{26,27}. Indeed, in the absence of extracellular calcium, capsaicin-evoked responses in VR1-transfected cells showed little or no desensitization during prolonged agonist application or with successive agonist challenges ($4.7 \pm 2.3\%$ decrease between first and third applications, $n = 3$ (Fig. 3c)). In contrast, responses evoked in calcium-containing bath solution consisted of at least two distinct components, one desensitizing ($87 \pm 4.3\%$ decrease between first and third applications, $n = 5$) and one relatively non-desensitizing. Thus desensitization and multiphasic kinetics of vanilloid-evoked responses can be reproduced without a neuronal context and can be distinguished by their dependence on ambient calcium levels.

The behaviour of the VR1 response was also examined in membrane patches excised from transfected cells. In the presence



c

rat VR1
human T12251
Calliphora z80230
Drosophila TRP
Bovine x99792
C. elegans z72508

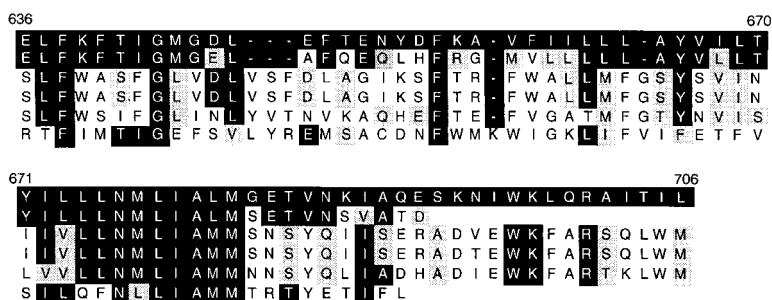


Figure 5 VR1 resembles store-operated channels. **a**, Predicted amino-acid sequence encoded by the vanilloid receptor cDNA VR1. Open boxes delineate ankyrin repeat domains, black boxes predicted transmembrane domains, the grey box a possible pore-loop, and filled circles predicted protein kinase A phosphorylation sites. **b**, Predicted membrane topology and domain structure of VR1. Outer (o) and inner (i) plasma membrane leaflets are indicated. **c**, Alignment of VR1 with related sequences. Identical residues are in black boxes and conservative substitutions are in grey. A partial sequence from a human EST is shown. Other sequences are from members of the putative store-operated calcium channel family. The Genbank accession number of the *Drosophila* TRP protein is p19334; others are indicated.

of capsaicin (but not its absence), large and well-resolved currents of unitary amplitude were observed ($n = 31$; Fig. 3d, e), indicating the existence of capsaicin-gated ion channels within these patches whose activation does not depend upon soluble cytoplasmic components. The current-voltage relation at the single-channel level was almost identical to that established in whole-cell configuration, owing to its outward rectification and reversal potential near 0 mV under similar ionic conditions (Fig. 3f). Unitary conductances of 76.7 pS at positive potentials and 35.4 pS at negative potentials were observed with sodium as the sole charge carrier. These single-channel properties are like those previously described for native vanilloid receptors^{8,28}. It has been suggested that the site of vanilloid action may not be confined to the extracellular side of the plasma membrane, owing to the lipophilic nature of these compounds⁶. We found that capsaicin was able to produce identical responses when added to either side of a patch excised from a cell expressing VR1 (Fig. 3e), consistent with the notion that vanilloids can permeate or cross the lipid bilayer to mediate their effects. A less likely but formally consistent explanation is that vanilloid receptors have functionally equivalent capsaicin-binding sites on both sides of the plasma membrane.

Capsaicin kills cells that express VR1

Capsaicin is an excitatory neurotoxin that selectively destroys primary afferent nociceptors *in vivo* and *in vitro*^{5,9}. Is this selective toxicity solely a reflection of the specificity of vanilloid receptor expression, or does it depend on additional properties of sensory neurons or their environment? To address this question, we sought to determine whether capsaicin could kill non-neuronal cells that express vanilloid receptors *in vitro*. We found that, within several hours of continuous exposure to capsaicin, HEK293 cells transfected with VR1 died, as determined morphologically and by staining with vital dyes (Fig. 4). In contrast, cells transfected with

vector alone were not killed by this treatment. The cell death was characterized by prominent cytoplasmic swelling, coalescence of cytoplasmic contents, and eventual lysis. Thus VR1 expression in a non-neuronal context can recapitulate the cytotoxicity observed in vanilloid-treated sensory neurons. Staining with Hoechst dye 33342 revealed no evidence of the nuclear fragmentation often associated with apoptotic cell death²⁹ (not shown). Together, these observations are consistent with necrotic cell death resulting from excessive ion influx, as has been proposed for vanilloid-induced death of nociceptors⁷, glutamate-induced excitotoxicity³⁰, and neurodegeneration caused by constitutively activating mutations of various ion channels³¹.

VR1 resembles TRP-related ion channels

The VR1 cDNA contains an open reading frame of 2,514 nucleotides that encodes a protein of 838 amino acids with a predicted relative molecular mass of 95,000 (M_r 95K) (Fig. 5a). Hydrophilicity analysis suggests that VR1 is a polytopic protein containing six transmembrane domains (predicted to be mostly β -sheet) with an additional short hydrophobic stretch between transmembrane regions 5 and 6 (Fig. 5b). The amino-terminal hydrophilic segment (432 amino acids) contains a relatively proline-rich region followed by three ankyrin repeat domains. The carboxy terminus (154 amino acids) contains no recognizable motifs.

A homology search of protein databases revealed significant similarities between VR1 and members of the family of putative store-operated calcium channels (SOCs), the prototypical members of which include the *Drosophila* retinal proteins TRP and TRPL^{32,33} (Fig. 5c). Members of this family have been proposed to mediate the entry of extracellular calcium into cells in response to the depletion of intracellular calcium stores³⁴. These proteins resemble VR1 with respect to their predicted topological organization and the presence of multiple N-terminal ankyrin repeats³³. There is also striking

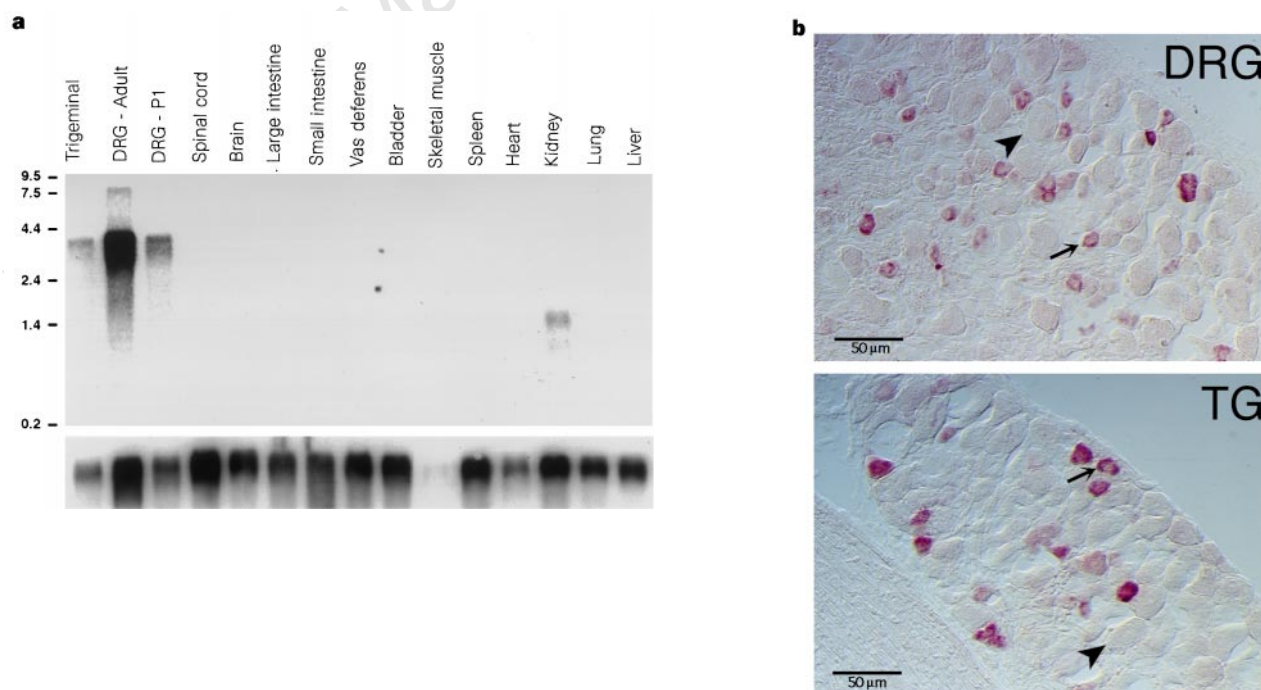


Figure 6 Vanilloid receptor expression is restricted to sensory neurons. **a**, Northern blot analysis shows that VR1 transcripts are confined to sensory ganglia. Poly(A)⁺ RNAs were prepared from adult rats, except for the DRG-P1 sample, which was isolated from dorsal root ganglia of newborn pups. The blot was probed with ³²P-labelled VR1 cDNA, then re-probed with a ³²P-labelled cyclophilin cDNA to control for loading (bottom). Molecular size markers

(kilobases) are shown on the left. **b**, *In situ* hybridization detects VR1 expression in a subset of sensory ganglion cells. Adult rat dorsal root ganglia (DRG) and trigeminal ganglia (TG) were probed with a digoxigenin-labelled, VR1-derived antisense riboprobe. Positive staining (purple) was confined to smaller-diameter cell bodies (arrows) and absent from large-diameter cell bodies (arrowheads). Control (sense) riboprobes did not stain these tissues (not shown).

amino-acid sequence similarity between VR1 and TRP-related proteins within and adjacent to the sixth transmembrane region, including the short hydrophobic region between transmembrane domains 5 and 6 that may contribute to the ion permeation path³³. Outside these regions, VR1 shares little sequence similarity with TRP family members, suggesting that its evolutionary relationship to these proteins is distant. Given the high permeability of VR1 to calcium ions, we nonetheless considered the possibility that it might function as a SOC. To test this, we examined calcium-dependent inward currents in VR1-expressing oocytes whose intracellular calcium stores had been depleted by treatment with the compound thapsigargin. In water-injected control oocytes, a clear depletion-induced current was seen, as previously described³⁵ (not shown). In VR1-expressing oocytes, no quantitative or qualitative differences were observed in this response (not shown). Moreover, application of SKF 96365 (20 μ M), an inhibitor of depletion-stimulated calcium entry³⁶, had no effect on capsaicin-evoked currents in VR1-expressing oocytes (not shown). Thus VR1 does not seem to be a functional SOC under these circumstances.

An expressed sequence tag (EST) database homology search revealed several human clones with a high degree of similarity to VR1 at both the nucleotide and predicted amino-acid levels. The similarity of one of these clones to the corresponding region of VR1 (Fig. 5c) is extremely high (68% amino-acid identity and 84% similarity within the region shown), suggesting that it is likely to be the human VR1 orthologue or a closely related subtype. Human EST clones corresponding to other domains of VR1 show comparable degrees of similarity (not shown), and could represent fragments of the same human transcript.

Sensory neuron-specific expression of VR1

The highly selective nature of capsaicin action suggests that vanilloid receptors serve as specific molecular markers for nociceptive neurons. Indeed, northern blot analysis showed that a mRNA species of approximately 4 kb is prominently and exclusively

expressed in trigeminal and dorsal root sensory ganglia, both of which contain capsaicin-sensitive neurons (Fig. 6a). This transcript was absent from all other tissues examined, including spinal cord and brain. A much smaller RNA species ($\sim$ 1.5 kb) was detected in the kidney, but it is unclear whether this transcript could encode a functional VR1 protein. *In situ* hybridization histochemistry was used to assess the cellular pattern of VR1 expression within sensory ganglia (Fig. 6b). These experiments clearly show that within dorsal root and trigeminal ganglia, VR1 expression predominates in a subset of neurons with small diameters. This is in keeping with the observation that most capsaicin-sensitive neurons have cell bodies of relatively small to medium size^{5,27}. In contrast to the prominent expression of VR1 transcripts in neurons of the dorsal root ganglion, no visible signal was observed in the adjacent spinal cord dorsal horn (not shown). Although binding sites for radiolabelled resiniferatoxin have been detected in the dorsal horn, they are believed to reside on presynaptic terminals that project from primary nociceptors with cell bodies located in the dorsal root ganglia²⁷. Our results support this interpretation. Two other tissues that have been proposed to express capsaicin receptors are the nodose ganglion, which contains cell bodies of visceral nociceptors²⁷, and the preoptic area of the hypothalamus², which is involved in thermoregulation³⁷. By using *in situ* hybridization methods, we did not detect VR1 expression at either location. Although these tissues might express VR1 at levels below the detection limit of our assay, vanilloid responsiveness here might be conferred by distinct VR subtypes. Indeed, VR heterogeneity has been proposed on the basis of biochemical studies^{4,16}.

Vanilloid receptor activated by noxious heat

The 'burning' quality of vanilloid-induced pain suggests that vanilloids and heat may evoke painful responses through a common molecular pathway. We therefore explored the effects of elevated temperature on VR1 activity. In initial studies, transfected HEK293 cells were subjected to fluorescent calcium imaging during

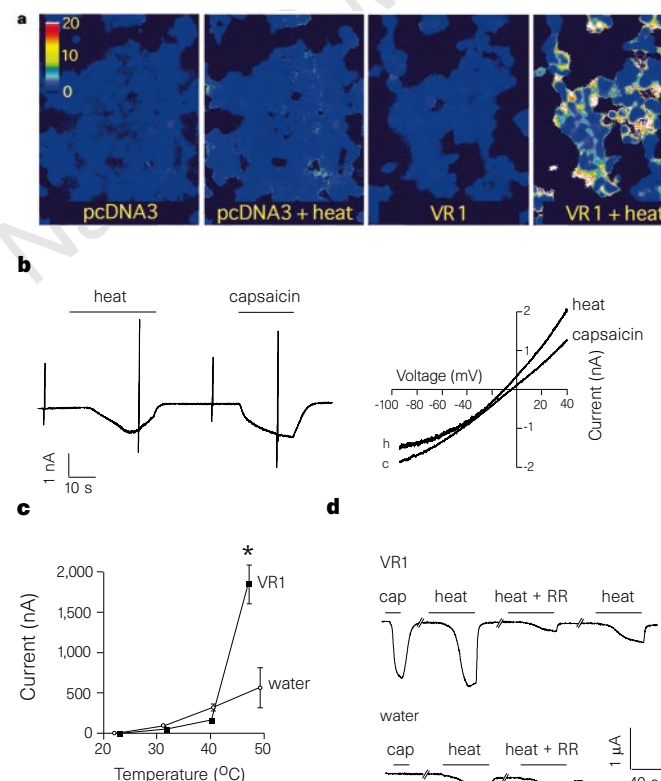


Figure 7 VR1 is activated by noxious thermal stimuli. **a**, HEK293 cells transiently transfected with VR1, but not vector alone (pcDNA3), exhibit a pronounced increase in cytoplasmic free calcium in response to heat. Cells were analysed before and immediately after addition of heated buffer (300 μ l CIB at 65°C was applied to cells in 150 μ l CIB at 22°C). Under these conditions, cells were transiently exposed to a peak temperature of $\sim$ 45°C. Relative calcium concentrations are indicated by the colour bar, as in Fig. 1. **b**, Whole-cell patch-clamp analysis ($V_{\text{hold}} = -60$ mV) of VR1-transfected HEK293 cells reveals inward current responses to both heat and capsaicin. The temperature of the bath medium was raised from 22 to 48°C (heat), and then restored to 22°C, after which capsaicin (0.5 μ M) was added to the bath (left trace). Ionic conditions were identical to those described in Fig. 3a. Voltage ramps (-100 to $+40$ mV in 500 ms) were applied before, between and during responses. Stimulus-induced current-voltage relations are shown on the right. **c**, VR1 expressed in *Xenopus* oocytes is activated by noxious but not innocuous heat. Oocytes injected with either VR1 cRNA or water were subjected to two-electrode voltage-clamp while the perfusate temperature was raised from 22.7°C to the level indicated, then held constant for 60 s. The magnitudes of the resulting inward currents are shown as the mean $\pm$ s.e.m. (VR1, $n = 8$; water, $n = 6$ independent cells). The asterisk indicates a significant difference from water-injected oocytes (t -test, $P < 0.0005$). **d**, Ruthenium red (RR) inhibits heat-evoked responses in VR1-expressing oocytes. The current tracings shown were generated from representative VR1- or water-injected oocytes during successive applications of the indicated stimuli. VR1-injected oocytes exhibited the following mean inward current responses $\pm$ s.e.m. ($n = 5$): capsaicin (cap, 1 μ M), $1,221 \pm 148$ nA; heat (50°C), $2,009 \pm 134$ nA; heat plus RR (10 μ M), 243 ± 47 nA. Inhibition by RR was significant ($88 \pm 2\%$, $n = 5$; paired t -test, $P < 0.0001$). In the absence of RR, no diminution in current was observed with successive heat pulses (not shown). Water-injected oocytes showed no response to capsaicin and much smaller responses to heat (338 ± 101 nA, $n = 5$). RR inhibited these responses by only $21 \pm 26\%$ ($n = 5$; paired t -test, $P < 0.1$).

a sudden increase in ambient temperature from 22 °C to ~45 °C. Under these conditions, cells transfected with vector alone exhibited only a mild, diffuse change in cytoplasmic free calcium (Fig. 7a, left). In contrast, a large proportion of cells expressing VR1 exhibited a pronounced increase in calcium levels within seconds of heat treatment (Fig. 7a, right). These responses subsided within a few minutes, and a subsequent challenge with capsaicin produced a characteristic calcium response (not shown), suggesting that the response to heat is a specific signalling event and not a consequence of nonspecific membrane perturbation or disruption of cell integrity. To determine whether specific heat-evoked membrane currents are associated with this phenomenon, VR1-expressing cells were examined using patch-clamp methods. Exposure of these cells to a rapid increase in temperature (22 °C to ~48 °C in 25 s, monitored using an in-bath thermistor) produced large inward currents (791 ± 235 pA at -60 mV, $n = 9$) that were typically similar in amplitude to that evoked by a subsequent application of capsaicin at 500 nM (Fig. 7b). Both heat- and vanilloid-evoked responses showed outward rectification, suggesting that they are mediated by the same entity (Fig. 7b). By comparison, thermally evoked responses of control, vector-transfected cells were much smaller (131 ± 23 pA, $n = 8$) and exhibited no rectification (not shown). The heat response in VR1-transfected cells desensitized during stimulus application, whereas that observed in vector-transfected cells did not. These results suggest that VR1 is acting as a thermal transducer, either by itself or in conjunction with other cellular components.

To determine whether VR1 could mediate similar responses to heat in a different cellular environment, we extended these studies to the oocyte system. In control, water-injected oocytes, acute elevation of perfusate temperature produced a small inward current that increased linearly up to 50 °C (Fig. 7c). VR1-expressing oocytes exhibited similar responses at temperatures up to 40 °C, but above this threshold their responses were significantly larger than those of controls. Thus, even in this non-mammalian context, VR1 expression confers heat sensitivity with a temperature-response profile that is remarkably consistent with that reported for thermal nociceptors¹. Pharmacological experiments also suggest that VR1 is involved directly in this thermal response: application of ruthenium red reduced significantly ($88 \pm 2\%$, $n = 5$) the response of

VR1-expressing oocytes to heat, whereas the smaller response seen in control cells was reduced by only $21 \pm 26\%$ ($n = 5$; Fig. 7d). Taken together, these observations strongly support the hypothesis that VR1 is activated by noxious, but not innocuous, heat.

Protons may be endogenous modulators of VR1

A reduction in tissue pH resulting from infection, inflammation or ischaemia can produce pain in mammals. It has therefore been proposed that protons might act as endogenous activators or modulators of vanilloid receptors^{38–40}. To address this possibility, we examined the effects of hydrogen ions on the cloned vanilloid receptor using the oocyte expression system. We investigated whether an abrupt reduction in bath solution pH, from 7.6 to 5.5, was sufficient to activate VR1 in the absence of capsaicin. Fewer than 10% of VR1-expressing oocytes treated in this way exhibited a large inward current (not shown), suggesting that hydrogen ions alone cannot efficiently activate this protein. We next assessed the effect of reduced pH on the responsiveness of VR1 to capsaicin. VR1-expressing oocytes were treated with a submaximal concentration of capsaicin (300 nM) at pH 7.6 (Fig. 8). Once their current responses reached a relatively stable plateau, the oocytes were exposed to a solution containing the same concentration of capsaicin at pH 6.3. Under these conditions, the inward current rapidly increased to a new plateau up to fivefold greater in magnitude than the first. Upon returning to pH 7.6, the oocyte response subsided to its initial plateau, and upon the removal of capsaicin it returned to baseline. This potentiation was seen only with subsaturating concentrations of agonist, as reduced pH did not augment responses to 10 μ M capsaicin (not shown). These results suggest that, although hydrogen ions alone are not sufficient to activate VR1, they can markedly potentiate capsaicin-evoked responses, presumably by increasing capsaicin potency.

Discussion

Opioids and vanilloids are natural products whose physiological effects are apparently so seductive or desirable that their use has permeated diverse cultures for thousands of years. Consequently, it has long been assumed that their molecular modes of action must involve important physiological processes that underlie endogenous mechanisms of pain sensation and regulation. In the study of opioid action, this assumption led to the discovery of the principal signalling system that suppresses pain *in vivo*⁴¹. We expected that the identification of the biological target of vanilloid action would similarly illuminate a fundamental mechanism of pain production. By pursuing this hypothesis, we have defined a molecular component of the nociceptive pathway that transduces two of the principal types of painful stimuli: thermal and chemical.

The cloning of VR1 demonstrates unequivocally that the molecular target of capsaicin action on sensory neurons is a proteinaceous ion channel. VR1 is structurally related to the TRP family of ion channels that have been proposed to mediate the influx of extracellular calcium in response to depletion of intracellular calcium stores. The precise physiological roles and mechanisms of activation of these channels have been the subject of much debate³⁴. It has been proposed that these channels are gated by diffusible small molecules released from depleted intracellular calcium stores, or alternatively that gating involves direct allosteric interactions with store-associated proteins. If TRP-related channels are gated by diffusible molecules, these molecules might bind to a site analogous to that used by vanilloid compounds to activate VR1. Indeed, SKF 96365, an inhibitor of depletion-mediated calcium entry, contains two methoxyphenyl moieties³⁶, which resemble vanillyl groups. An understanding of VR1 activation may therefore provide new molecular insights into these broader biochemical issues. In any case, the distant but clear molecular relationship between VR1 and TRP family members makes it likely that this group of ion channels subserves diverse physiological functions.

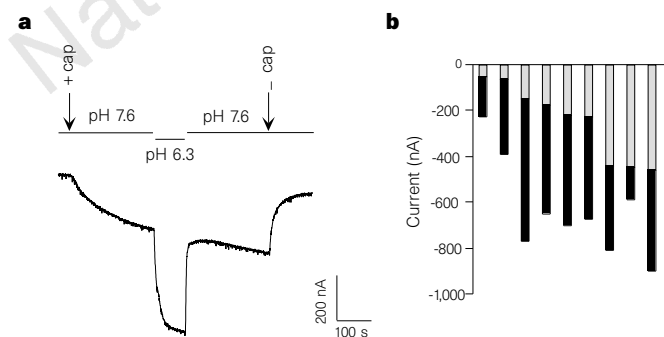


Figure 8 Hydrogen ions potentiate the effect of capsaicin on VR1. **a**, Augmentation of capsaicin-evoked current response by reduced pH in VR1-expressing oocytes. Capsaicin (cap, 0.3 μ M) was administered throughout the time period spanned by the arrows. The pH of the bath solution was changed during the experiment as indicated by the horizontal bars. VR1-expressing oocytes exhibited no responses to pH 6.3 bath solution without capsaicin; water-injected control oocytes exhibited no responses to either capsaicin or pH 6.3 bath solution (not shown). **b**, Summary of current responses obtained from 9 independent VR1-expressing oocytes. The grey portion of each bar indicates peak current evoked by capsaicin at pH 7.6; the black portion represents the additional current evoked by changing the pH to 6.3.

The cloned capsaicin receptor is activated not only by vanilloid compounds but also by thermal stimuli within the noxious temperature range. The temperature–response profile of VR1 matches very closely those reported for heat-evoked pain responses in humans and animals, and for heat-evoked currents in cultured sensory neurons^{1,42,43}. Several other properties of VR1-mediated heat responses are like those seen in whole animals or in cultured sensory neurons. For instance, ruthenium red, which blocks VR1 activity in mammalian cells or oocytes, also blocks heat-evoked nociceptive responses in the rabbit ear⁴⁴. In addition, the heat-evoked currents observed in both VR1-expressing cells and cultured sensory neurons are carried through outwardly rectifying, non-selective cation channels^{42,43}. Electrophysiological recordings from cultured sensory neurons reveal a striking concordance between responsiveness to capsaicin and heat⁴⁵. Our findings, together with these observations, suggest that an *in vivo* role of vanilloid receptors is to detect noxious heat. But not all characteristics of endogenous heat responses resemble those mediated by VR1. In the whole animal, capsaicin pretreatment reduces responsiveness to noxious thermal stimuli in some, but not all, physiological contexts^{2,3,5}. Similarly, in cultured sensory neurons, some heat-activated currents are reported to be insensitive to ruthenium red and to exhibit a lower relative contribution from calcium ions^{42,43}. Thus responses to noxious thermal stimuli may be transduced through multiple molecular pathways, only some of which may involve VR1. It is presently unclear whether thermosensitivity is an intrinsic physical property of VR1, but the fact that VR1 confers heat responsiveness to both mammalian cells and frog oocytes indicates that any other requisite component(s) must be widely expressed. Disruption of the VR1 gene in mice should help to clarify these issues.

The activation of VR1 by heat does not exclude the possibility that small molecules or other endogenous factors also modulate vanilloid-receptor function. It has been proposed, for instance, that protons produce pain by activating vanilloid receptors³⁸. Although we did not observe consistent activation of VR1 by protons alone, we did find that protons can potentiate vanilloid-evoked responses in VR1-expressing oocytes. This is consistent with the observation that hydrogen ions potentiate the effects of low concentrations of capsaicin on cultured sensory neurons^{39,40}. Moreover, in preliminary experiments, we have observed that low pH can also increase the response of VR1 to noxious thermal stimuli in the oocyte expression system. Thus the increased response to noxious stimuli (hyperalgesia) that accompanies inflammation and ischaemia might result, in part, from an enhancement of vanilloid receptor function by the excess hydrogen ions they produce³⁸. If this is the case, VR1 could provide an important model system for the *in vitro* study of hyperalgesia.

The multiple consequences of vanilloid receptor activation make it possible that this protein is involved in diverse human disease states ranging from congenital pain insensitivity to chronic pain syndromes. The cloning of VR1 provides both a molecular probe with which to address these possibilities and a defined target for the development of new analgesic agents. □

Methods

Expression cloning and DNA analysis. A rodent dorsal root ganglion plasmid cDNA library was constructed in pCDNA3 (Invitrogen) essentially as described⁴⁶, using a mixture of polyadenylated RNA from newborn rat and adult mouse dorsal root ganglia to generate first-strand cDNA. The resulting 2.4×10^6 independent bacterial clones were divided into 144 pools. HEK293 cells expressing the SV40 large T antigen (gift from T. Livelli) and maintained in DMEM (supplemented with 10% fetal bovine serum (Hyclone), penicillin, streptomycin and L-glutamine) were transfected with plasmid DNA from individual pools using a calcium phosphate precipitation kit (Specialty Media). The next day the cells were replated onto eight-well polyornithine-coated chambered coverglasses (Applied Scientific). Between 6 and 24 h later they were loaded with Fura-2 (30 min at 37°C) in CIB buffer containing (in

mM) 130 NaCl, 3 KCl, 2.5 CaCl₂, 0.6 MgCl₂, 1.2 NaHCO₃, 10 glucose, 10 HEPES, pH 7.45, with 10 μM Fura-2 acetoxymethyl ester and 0.02% pluronic acid (Molecular Probes), then rinsed twice with CIB. Ratiometric calcium imaging was performed using a Nikon Diaphot fluorescence microscope equipped with a variable filter wheel (Sutter Instruments) and an intensified CCD camera (Hamamatsu). Dual images (340 and 380 nm excitation, 510 nm emission) were collected and pseudocolour ratiometric images monitored during the experiment (Metafluor software, Universal Imaging). Cells were initially imaged in 200 μl CIB, after which 200 μl CIB containing capsaicin at twice the desired concentration was added. After stimulation, cells were observed for 60–120 s. For each library pool, one microscopic field (300–500 cells) was assayed in each of eight wells. DNA sequencing was performed using an automated sequencer (ABI). Homology searches were performed against the non-redundant Genbank database and against an EST database (dbEST). Hydrophilicity was calculated using the Hopp–Woods algorithm⁴⁷. VR1 was determined to be of rat origin by sequencing an independent cDNA isolated from a rat dorsal root ganglia library and a polymerase chain reaction (PCR) product derived from mouse dorsal root ganglia.

Oocyte electrophysiology. cRNA transcripts were synthesized from *Not1*-linearized VR1 cDNA templates using T7 RNA polymerase⁴⁶. Defolliculated *Xenopus laevis* oocytes were injected with 0.5–5 ng VR1 cRNA. Four to seven days after injection, two-electrode voltage-clamp recording was performed ($E_{\text{hold}} = -60$ mV for IC₅₀ curve and thermal stimulation experiments, and -40 mV for all other experiments) using a Geneclamp 500 amplifier (Axon Instruments) and a MacLab A/D converter (MacLab). The recording chamber was perfused at a rate of 2 ml min⁻¹ with frog Ringer's solution containing (in mM) 90 NaCl, 1.0 KCl, 2.4 NaHCO₃, 0.1 BaCl₂, 1.0 MgCl₂ and 10 HEPES, pH 7.6, at room temperature. CaCl₂ (2 mM) was used instead of BaCl₂ when generating the capsazepine inhibition curve. Thermal stimuli were applied using a preheated bath solution and temperature was monitored using a thermistor placed next to the oocyte. For store-operated current assays, oocytes were incubated for 1–2 h in calcium-free, barium-free frog Ringer's solution containing 1 mM EGTA and 1 μM thapsigargin. During voltage-clamp recording, these oocytes were intermittently exposed to frog Ringer's solution containing 2 mM Ca²⁺ and no EGTA to detect calcium-dependent currents (15-s pulses at 2-min intervals)³⁵. Capsazepine, ruthenium red and thapsigargin were purchased from RBI, SKF 96365 from ICN, and all other chemicals from Sigma. Finely chopped whole peppers (15 g) were extracted overnight at room temperature with 50 ml absolute ethanol. Soluble extracts were concentrated 15-fold by vacuum desiccation, then diluted 1,000-fold in frog Ringer's solution for electrophysiological assay.

Mammalian cell electrophysiology. Patch-clamp recordings were performed with transiently transfected HEK293 cells at 22°C. Standard bath solution for whole-cell recordings contained (in mM) 140 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES, 10 glucose, pH 7.4 (adjusted with NaOH). In calcium-free bath solution, CaCl₂ was replaced with 5 mM EGTA. For monovalent cation substitution experiments, after the whole-cell configuration was obtained in standard bath solution, the bath solution was changed to (in mM) 140 NaCl (or KCl or CsCl), 10 glucose and 10 HEPES (adjusted to pH 7.4 with NaOH, KOH or CsOH, respectively) and the reversal potential measured using voltage ramps (see Fig. 3 legend). For divalent cation permeability experiments, the bath solution was changed to (in mM) 110 MgCl₂ (or CaCl₂), 2 Mg(OH)₂ (or Ca(OH)₂), 10 glucose, 10 HEPES, pH 7.4 (adjusted with HCl). Bath solution for outside-out patch recordings and pipette solution for inside-out patch recordings contained (in mM) 140 NaCl, 10 HEPES, pH 7.4 (adjusted with NaOH). Bath solution for inside-out patch recordings and pipette solutions for outside-out patch recordings and ion substitution experiments contained (in mM) 140 NaCl, 10 HEPES, 5 EGTA, pH 7.4 (adjusted with NaOH). Pipette solution for other whole-cell recordings contained (in mM) 140 CsCl (or 130 CsAspartate and 10 NaCl), 5 EGTA, 10 HEPES, pH 7.4 (adjusted with CsOH). Liquid junction potentials were measured directly in separate experiments; they did not exceed 3 mV with solutions used and no correction for this offset was made. Whole-cell recording data were sampled at 20 kHz and filtered at 5 kHz for analysis (Axopatch 200 amplifier with pCLAMP software, Axon Instruments). Single-channel recording data were sampled at 10 kHz and filtered at 1 kHz. Permeability ratios for monovalent cations to Na (P_X/P_{Na}) were calculated as follows: $P_X/P_{\text{Na}} = \exp(\Delta V_{\text{rev}}/FRT)$, where V_{rev} is the reversal

potential, F is Faraday's constant, R is the universal gas constant, and T is absolute temperature. For measurements of divalent permeability, P_Y/P_{Na} was calculated as follows: $P_Y/P_{Na} = [Na^+]_i \exp(\Delta V_{rev} F/RT) / (1 + \exp(\Delta V_{rev} F/RT)) / 4[Y^{2+}]_o$ (ref. 48), where the bracketed terms are activities. Assumed ion activity coefficients are 0.75 for monovalents and 0.25 for divalents⁴⁸.

Cell death, northern blot, and *in situ* hybridization analyses. For cell death assays, HEK293 cells were transfected as described above. Cells were rinsed twice with PBS 14 h later and re-fed with medium containing either capsaicin (3 μ M) or vehicle alone (ethanol, 0.3% final). After 7 h, cells were collected and the number of dead cells quantified with an ethidium homodimer and a calcein green-containing cell viability kit (Molecular Probes). For northern analysis, rat-derived poly(A)⁺ RNA was purified as described⁴⁹ or with the FastTrack kit (Invitrogen). Approximately 2 μ g of each sample was electrophoresed through a 0.8% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham), and hybridized at high stringency with a ³²P-labelled probe representing the entire VR1 cDNA. For *in situ* hybridization histochemistry, adult female Sprague-Dawley rats were anaesthetized and perfused with 4% paraformaldehyde in PBS. Tissues were dissected, frozen in liquid N₂ and embedded in OCT mounting medium. Cryostat sections (15 μ m thick) were processed and probed with a digoxigenin-labelled cRNA generated by *in vitro* transcription of a 1-kb fragment of the VR1 cDNA (nucleotides 1513–2482) (Genius kit, Boehringer Mannheim). Sections were developed with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments according to the manufacturer's instructions.

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Ambient acoustic imaging in helioseismology

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The increasing availability of high spatial resolution data of velocity and intensity variations on the Sun has stimulated the development of helioseismological techniques that probe the solar interior in localized regions. The techniques developed so far^{1–4} have yielded information on physical quantities (such as the flow velocity and magnetic field) below the surface, but are still far from providing a detailed picture of local subsurface inhomogeneities. Here we report the development and application of a new method for constructing three-dimensional solar images, utilizing acoustic noise (or stochastic P-mode oscillations) in the Sun. We treat a region of the solar surface as a phased array of acoustic sensors, which acts as a computational ‘lens’; acoustic waves ‘scattered’ by local inhomogeneities, such as sunspots, are collected and summed in phase, based on the knowledge of how (on average) they travel within the Sun. In this way, we are able to construct a three-dimensional image of a region of the solar interior.

The motivation for this acoustic imaging technique is our normal experience of imaging objects with ambient light, coupled with the recent imaging of objects with ambient acoustic noise in the ocean⁵. In the case of light imaging, the object is illuminated by scattered daylight, and its image is formed by an optical lens. The function of the optical lens is to add the electromagnetic signals from each point on the target object in phase to form an image. The solar acoustic waves (P-mode waves), which are continually generated and dissipated stochastically by the turbulent convection, play the same role as ambient light. Local inhomogeneities in the Sun, such as sunspots, scatter and absorb acoustic waves, and this is analogous to the way that dark transparent objects scatter and absorb ambient light. The main differences for the acoustic waves in the Sun are: (1) instead of propagating in a straight line, the ray paths of solar acoustic waves are strongly curved by the increase of sound speed with depth; (2) acoustic waves can be trapped and multiply reflected in an acoustic cavity between the surface and a layer in the solar interior; (3) we cannot insert an ‘acoustic lens’ to phase-match the acoustic signals from a target point in the Sun as an optical lens does for electromagnetic signals on Earth or a parabolic reflecting dish does for acoustic signals in the ocean.

In helioseismic ambient acoustic imaging, we form a ‘computational acoustic lens’. Each point on the solar surface has a velocity and intensity variation caused by the acoustic waves impinging upon it. We can use a spatial array of solar locations as the elements of an acoustic phased array. The acoustic wavetrain originating from a target point at the surface propagates inwards to the bottom of the acoustic cavity and back to the surface at a distance from the target point³. Different P-modes have different paths and arrive at the surface at different times and different distances from the target point. On the basis of the relation between travel time and distance travelled by acoustic waves in the Sun, we can phase-shift the time series of oscillation signals over the array to coherently detect the intensity of the acoustic signals from a target point in the Sun. Although the P-mode signal at each point in the collecting area

includes waves from all other points in the Sun, those signals are expected to cancel in the summation because their phases have different spatial coherence. Other local helioseismic techniques indirectly infer the subsurface structure by inverting the measured frequency shift, absorption, phase shift and time delay, whereas ambient acoustic imaging can directly image the three-dimensional inhomogeneities below the surface. A mathematical description of a similar concept has been presented elsewhere⁶.

We have used a seven-day helioseismic time series, totalling 90 hours of observations, taken with the Taiwan Oscillation Network (TON)⁷ to reconstruct a solar acoustic image at the surface. Images of the intensity of the Ca II K-line were taken during the interval 21–27 June 1996, and the sunspot region NOAA 7973 was selected as a test target. The data reduction procedure is as follows. (1) Each observed K-line full-disk image is transformed into coordinates of $\sin \theta$ and ϕ , where θ and ϕ are the latitude and the longitude, respectively, in a spherical coordinate system aligned along the solar rotation axis. (2) The differential rotation of the solar surface is

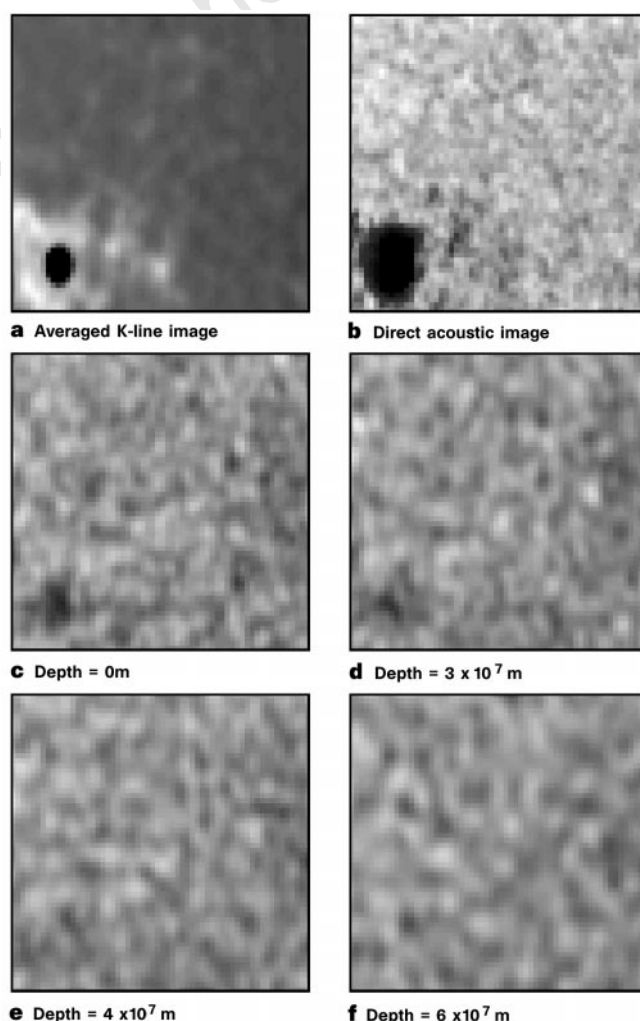


Figure 1 **a** K-line image; **b**, direct acoustic image; and **c–f**, reconstructed acoustic images at various depths for the same area. The horizontal (ϕ) and vertical ($\sin \theta$) dimensions are 21.1° and 13.9° , respectively. The K-line intensity image in **a** is averaged over the 7 days of observation. The blurred plage structure is caused by evolution during the observing interval. The constructed acoustic intensity images at the surface and at depths of 3×10^7 , 4×10^7 and 6×10^7 m are shown in **c**, **d**, **e** and **f** respectively. The suppression of acoustic intensity in the sunspot region is clear at the surface, and decreases with depth. At 4×10^7 m below the surface, the signature of sunspot disappears. The spatial fluctuation in the quiet Sun in **c–f** is believed to be noise, discussed in the text.

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removed. (3) The oscillatory amplitude is computed by subtracting the 15-frame running mean from the intensity time series at each spatial point. (4) A temporal filter is applied to isolate the signal in a range of 2.7–6.5 mHz. (5) The signal originating from a target point at time t is phase-matched by summing the signals at different angular distances from the target point measured at the appropriate time delays based on the one-bounce time–distance relation obtained from the quiet Sun³. The signals are summed over an annular region 2–26° from the target point, corresponding to travel times of 31–80 minutes after t . (6) The phased summation is repeated for each t in the time series, and then squared amplitudes at different times are added to obtain the acoustic intensity received from the target point. (7) The procedure is repeated for all points in a target region to form a two-dimensional acoustic image. We have applied the time–distance relation of the quiet Sun to both quiet and magnetic regions. The presence of magnetic field in the active region introduces a small change in the total travel time (about 0.2 minutes⁸) which is much smaller than the period of P-modes (about 5 minutes). Moreover, it should be noted that the differential travel time, which is the difference in travel time of two adjacent points on the time–distance curve, enters our phase-matching technique instead of the total travel time. The change in the differential travel time due to magnetic field is expected to be much smaller than the change in the total travel time. Thus we expect that the presence of magnetic field has little effect on our result, although further investigation is needed. Choosing an annular region surrounding the target point as the phased array means that our images display the azimuthally averaged acoustic intensity.

The reconstructed acoustic image at the surface is shown in Fig. 1c. For comparison, Fig. 1a shows the corresponding image for the average observed K-line intensity, and Fig. 1b shows the corresponding direct acoustic image computed by summing the squared amplitude of acoustic waves measured in the target region. The sunspot umbra is masked in the direct image, because scattered light contaminates the measurement. The correspondence between the reconstructed image, Fig. 1c, and the direct image, Fig. 1b, is apparent inside and near the sunspot where the suppression of acoustic intensity is strong. The correspondence is not clear in the plage regions where the suppression is weak.

The acoustic intensity reconstructed here is the power of acoustic waves propagating outwards from the target points. We have also reconstructed an image of incoming waves with a time-reversed time–distance relation, which, as expected, does not show any signature of the sunspot because the incoming waves have not been affected by the sunspot before reaching it. The fraction of the

acoustic intensity suppressed in Fig. 1c relates to the absorption integrated along the ray paths from the target point. We believe that the main component of the spatial fluctuation of intensity in the quiet Sun shown in Fig. 1c is noise because its fractional amplitude σ/μ is inversely proportional to the square root of the length of data series. Here μ is the acoustic intensity averaged over the quiet Sun in Fig. 1c (excluding the penumbra and umbra), and σ is the standard deviation of the acoustic intensity in the corresponding area. The source of the noise might be the incomplete cancellation of signals from points other than the target point, or the variation of the time–distance relation, or the variation of excitation and dissipation of P-modes. The suppression of acoustic intensity can be as large as 3.9σ inside the umbra. This new method can be tested by experiments with incorrect time–distance relations. The experiments show that images computed with incorrect time–distance relations do not show the signature of the sunspot, and have intensities significantly lower than those of Fig. 1, computed with the correct time–distance relation.

The goal of this acoustic imaging method is to construct a three-dimensional acoustic image of the solar interior. One application is in inferring the subsurface magnetic structure from the constructed images below the surface. Constructing the two-dimensional image at depth requires a time–distance relation for that depth. We computed the time–distance relations for a set of depths, using a standard solar model, based on the ray theory at 3 mHz. The time–distance relations we used to construct the acoustic images in Fig. 1 at various depths are shown in Fig. 2. Notice that the computed relation at the surface agrees well with the observed one⁹. The constructed images at depths of 3×10^7 , 4×10^7 and 6×10^7 m are shown in Fig. 1d, e and f, respectively. We have used the same annular region, 2–26°, for all depths. These subsurface acoustic images show several phenomena. (1) The suppression of acoustic wave intensity at the location of the sunspot disappears at a depth of about 4×10^7 m. The acoustic intensity averaged over the umbra and penumbra normalized by μ is shown in Fig. 3 as a function of depth. This result is consistent with the depth variation of the interaction parameter inferred from the inversion of measured absorption coefficients in sunspots¹⁰. (2) The location of the acoustic intensity minimum does not shift with depth. This implies that the magnetic flux tube corresponding to the sunspot is almost vertical down to a depth of at least 3×10^7 m. (3) The size of acoustic suppression region does not change as dramatically as the case of monolithic constant β (ratio of gas pressure to magnetic pressure) where the sunspot radius decreases very rapidly with depth. Instead, it favours Parker's fibril model¹¹ for sunspots. (4)

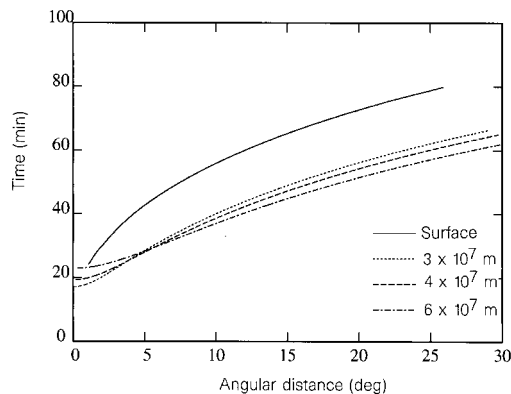


Figure 2 Time–distance relations at various depths, computed from a standard solar model, based on the ray theory at 3 mHz. These curves were used to construct the subsurface acoustic images in Fig. 1.

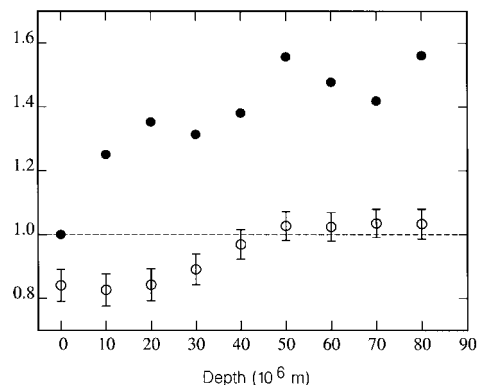


Figure 3 Relative mean quiet Sun acoustic intensity, μ (solid dots), defined in the text, and the average acoustic intensity inside the sunspot normalized by μ (open dots). The error bar is σ/μ , defined in the text. The mean acoustic intensity increases with depth. The fraction of acoustic intensity suppression inside the sunspot is about 16% at the surface. It decreases with depth, and vanishes at a depth of about 4×10^7 m.

The mean acoustic intensity, μ , in the quiet Sun seems to increase with depth, as shown in Fig. 3. The depth dependence of μ may be related to the variation of acoustic absorption with depth and the fraction of the acoustic spatiotemporal spectrum that is detected in viewing different depths. (5) The spatial resolution of constructed images decreases with depth. The time–distance relations flatten with increasing depth, so the phase gradient over the observing annulus decreases. The difference in phases used to image adjacent spatial points becomes small, and a point in space is imaged with a broad point-spread function. Another cause is that shorter-wave-length modes cannot be detected in viewing a deeper region.

Finally, we mention some important questions that call for further study. What is the degree of cancellation of signals from points other than the target point in this phased detection? What are the horizontal and vertical spatial resolutions corresponding to this ‘computational acoustic lens’? What is the correct interpretation of the mean intensity, μ , and its apparent variation with depth? Can this method reconstruct the complex index of refraction of acoustic waves at each target point, using the phase information in addition to the intensity? Finally, could one use this method to detect active regions before they emerge, or active regions at the other side of the Sun? □

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Capillary flow as the cause of ring stains from dried liquid drops

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When a spilled drop of coffee dries on a solid surface, it leaves a dense, ring-like deposit along the perimeter (Fig. 1a). The coffee—initially dispersed over the entire drop—becomes concentrated into a tiny fraction of it. Such ring deposits are common wherever drops containing dispersed solids evaporate on a surface, and they influence processes such as printing, washing and coating^{1–5}. Ring deposits also provide a potential means to write or deposit a fine pattern onto a surface. Here we ascribe the characteristic pattern of the deposition to a form of capillary flow in which pinning of the contact line of the drying drop ensures that liquid evaporating from the edge is replenished by liquid from the interior. The resulting outward flow can carry virtually all the dispersed material to the edge. This mechanism

predicts a distinctive power-law growth of the ring mass with time—a law independent of the particular substrate, carrier fluid or deposited solids. We have verified this law by microscopic observations of colloidal fluids.

Our qualitative observations show that rings form for a wide variety of substrates, dispersed materials (solutes), and carrier liquids (solvents), as long as (1) the solvent meets the surface at a non-zero contact angle, (2) the contact line is pinned to its initial position as is commonly the case, and (3) the solvent evaporates. In addition, we found that mechanisms typically responsible for solute transport—surface-tension gradients, solute diffusion, electrostatic and gravity effects—are negligible in ring formation. Based on these findings, we have identified the minimal ingredients for a quantitative theory. The phenomenon is due to a geometrical constraint: the free surface, constrained by a pinned contact line, squeezes the fluid outwards to compensate for evaporative losses. We first describe the main features and results of our theory and then show our experimental tests of its validity.

Figure 2 illustrates the factors leading to outward flow in a small, thin, dilute, circular drop of fixed radius R slowly drying on a solid surface. The evaporative flux $J(r)$ reduces the height $h(r)$ at every point r . If there were no flow, the evaporation would alter the height

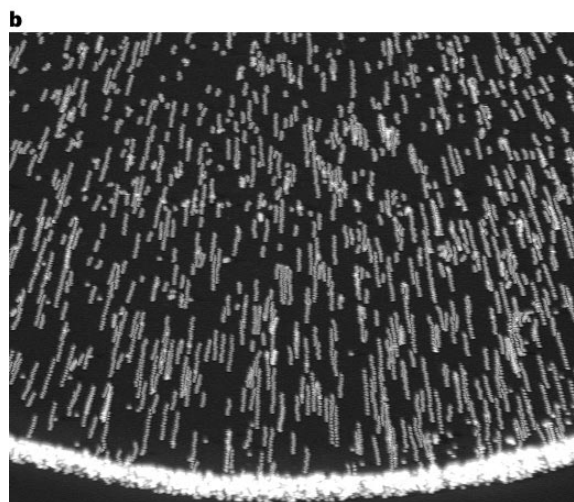
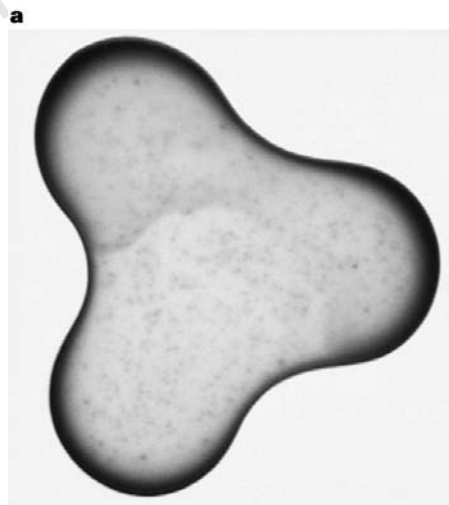


Figure 1 A ring stain and a demonstration of the physical processes involved in production of such a stain. **a**, A 2-cm-diameter drop of coffee containing 1 wt% solids has dried to form a perimeter ring, accentuated in regions of high curvature. **b**, Spheres in water during evaporation, as described in the text. Multiple exposures are superimposed to indicate the motion of the microspheres.

profile (Fig. 2a). At the perimeter, all the liquid would be removed and the drop would shrink. But the radius of the drop cannot shrink, as its contact line is pinned. To prevent the shrinkage, liquid must flow outwards as in Fig. 2b. The complete flow profile $\bar{v}(r)$ can be found, as shown in Fig. 2c. The height profile must maintain the spherical cap shape dictated by surface tension. Thus during a short time Δt , the region shown with vertical stripes in Fig. 2c must be removed from each point r of the surface. This is different from the amount removed from that point owing to evaporation, shown by the shaded regions in Fig. 2a. Radial flow must make up for this difference. If the evaporative flux $J(r)$ is known, the flow velocity $\bar{v}(r)$ is thereby determined.

The evaporative flux has a universal form that depends only on the shape of the drop (for example, see refs 6, 7). In the evaporation process liquid molecules interchange rapidly between the surface and the adjacent air, so that this air is saturated with vapour. As the air at infinity is not saturated, the vapour diffuses outward. At the surface of the drop (which is the only region where we need to calculate the evaporation current) the vapour quickly approaches a steady-state concentration profile $\phi(r)$ which obeys the steady-state diffusion equation $\nabla^2 \phi = 0$. At infinity, ϕ is the ambient concentration ϕ_∞ . At the drop surface ϕ is fixed at the saturation concentration ϕ_s . The derivative at the surface gives the desired evaporating flux $J(r) = -D\nabla\phi$, where D is the diffusivity of the vapour in air.

We may find the flux J by solving an equivalent electrostatic problem⁸, wherein ϕ is an electrostatic potential and the drop, with its fixed potential, is a conductor. (On the substrate surface beyond the drop there is no flux, so that the normal derivative of ϕ is zero there. That surface is in effect a reflection plane of symmetry, and

the opening angle of the conducting wedge is twice the contact angle that the drop makes with the substrate.) The sharp wedge-shaped boundary of the drop and its reflection leads to a diverging normal derivative (electric field or evaporative flux) as r approaches the contact line. For a drop with contact angle θ_c , this divergence has the form⁹: $J(r) \propto (R-r)^{-\lambda}$, where $\lambda = (\pi - 2\theta_c)/(2\pi - 2\theta_c)$. As the contact angle decreases towards zero, λ increases towards 1/2.

The initial deposition over times much shorter than the drying time depends sensitively on this diverging flux. To replace the diverging flux requires a diverging velocity near the perimeter: $\bar{v} \propto J \propto (R-r)^{-\lambda}$. We now consider the mass of solute, $M(r, t)$ beyond distance r from the centre at time t . Near the contact line the initial mass $M(r, 0)$ is proportional to the volume of this wedge-shaped region: $M(r, 0) \propto (R-r)^2$. All this mass will be entrained in the ring in the time t required for a point starting at r_t to move to the contact line:

$$t = \int_{r_t}^R dr/\bar{v} \propto (R-r_t)^{1+\lambda} \quad (1)$$

as $\bar{v} \propto (R-r)^{-\lambda}$. Substituting for r_t in terms of t into $M(r_t, 0)$ yields a power law for the early time growth of the ring: $M(R, t) = M(r_t, 0) \propto t^{2/(1+\lambda)}$.

At late times, the theory predicts complete transfer of the solute to the perimeter. As the time t approaches the drying time t_f , the height $h(r)$ decreases to zero as $(t_f - t)$. But, to leading order, the outward current $h(r)\bar{v}(r)$ must stay constant with time in order to replenish the constant evaporation flux J . Thus $\bar{v}$ must grow as $(t_f - t)^{-1}$. This diverging velocity leads to a diverging displacement of any point $r > 0$. Thus all initial points $r > 0$ are carried to the perimeter before the drying time t_f .

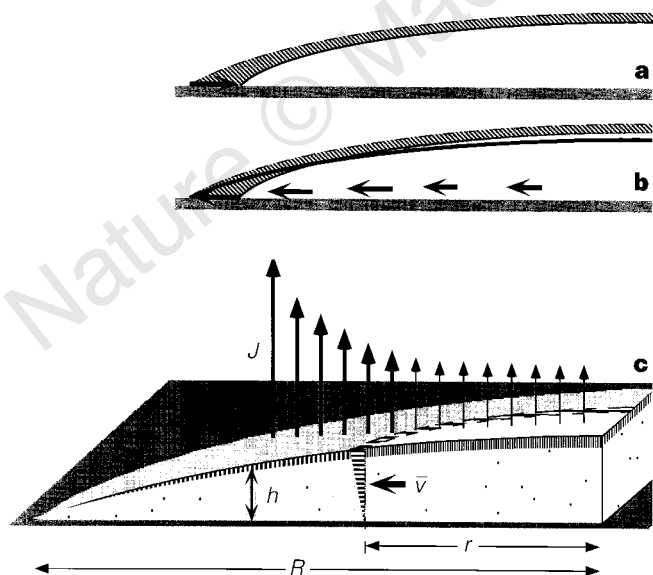


Figure 2 Mechanism of outward flow during evaporation. **a** and **b** show an increment of evaporation viewed in cross-section. **a**, The result of evaporation without flow: the droplet shrinks. **b**, The compensating flow needed to keep the contact line fixed. In **c**, we define the quantities responsible for flow. Vapour leaves at a rate per unit area $J(r)$. The removed liquid contracts the height $h(r)$ vertically, vacating the vertically striped region in a short time Δt . The volume of this striped region is equal to the volume removed by J . But in the shaded annular region the heavy-striped volume is smaller than the volume removed by J there (heavy arrows). Thus liquid flows outwards to supply the deficit volume: fluid at r sweeps out the horizontally striped region in time Δt . Its volume is the deficit volume; its depth-averaged speed is $\bar{v}(r)$.

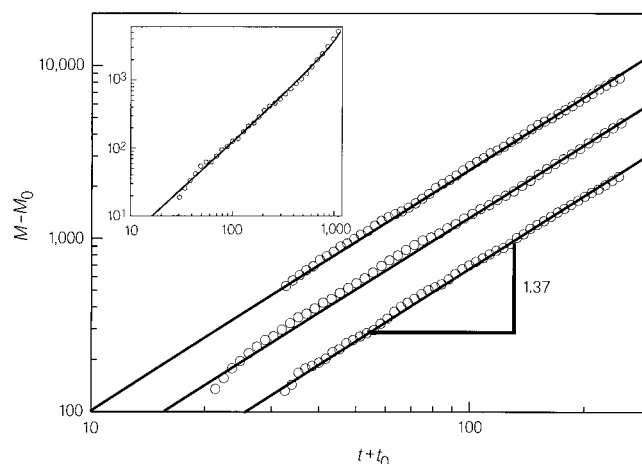


Figure 3 Double-logarithmic plot of ring mass $M(R, t)$ against time t for times from 10 to 250 seconds after placement of the drop on the surface, for three different drops whose total drying time was about 800 s. $M(R, t)$ is measured in number of particles. Particle number is counted in a sector of the ring similar to that shown in Fig. 1b. An offset $M_0 = 2$ was subtracted from the lowest curve to account for early non-steady-state deposition. $M_0 = 50$ and 40 for the middle and upper curves. The upper curve coincided with the middle one and was shifted up by a factor of 2 for clarity. An offset $t_0 = 11$ s, 13 s and 10 s was added to the time axis. The solid lines show the power law $(M - M_0) = \text{const} (t + t_0)^{1.37}$. Inset shows $M(R, t)$ versus t for the entire 1,300-s drying time. Circles: data obtained from 10-micrometre spheres via counting. Roughly 90% of the particles were observed to go to the contact line. Solid line: theoretical prediction determined numerically.

To test these predictions we used drops of distilled water which contained charge-stabilized surfactant-free polystyrene microspheres (Interfacial Dynamics, Portland, OR) at a starting volume fraction of 10^{-4} . The spheres were so dilute that they could be regarded as an ideal solution except in the very narrow region occupied by the ring. Droplets with nominal radius 2 mm were deposited on glass microscope slides and allowed to dry in a large enclosure with measured ambient temperature and humidity. The volume of the droplet was inferred by weighing the slide during drying. The volume decreased at a rate which agreed within 2% with that expected for steady-state vapour-diffusion-limited evaporation, using tabulated values¹⁰ for the water diffusivity in air and for the saturated vapour concentration.

In a separate experiment we observed the solute ring deposition by viewing the migration of 1- μm microspheres in a video microscope during drying (Fig. 1b). By automatically analysing¹¹ the video record, the depth-averaged velocity $\bar{v}(r)$ and the number of particles in the ring $M(R, t)$ were measured. Contact-line pinning is produced by surface irregularities and is much stronger with the solute than without it. The ring deposit can create surface unevenness as well as augment the surface imperfections that produced the initial pinning.

The depth-averaged velocity $\bar{v}(r)$ for thin drops, with $\theta_c \approx 0$, shows the predicted $(R - r)^{-0.5}$ divergence in the vicinity of the contact line. The measured ring mass $M(R, t)$ is plotted in Fig. 3. These drops had an initial contact angle $\theta_c \approx 0.25$ radians. For this angle, our theory predicts an initial increase $M(R, t) \approx t^{1.37}$. This behaviour is not expected at very early times ($t \ll (4R)^2/D \approx 10$ s), before steady-state diffusion has been achieved. To account for transient effects during this early period, we allow a shift in the effective starting time of the order of 10 s. We also include an offset in the deposited mass to account for the particles deposited during the initial transient. Choosing the M and t offsets to achieve the best straight line on a log-log plot yielded power-law fits $M - M_0 \approx (t + t_0)^p$, where $p = 1.3 \pm 0.1$. Thus the expected initial growth of the ring is consistent with observation. To predict the growth of the ring at later times, we first determine $\bar{v}(r)$ numerically from the known flux $J(r)$ of a thick drop and then use this $\bar{v}(r)$ to determine $M(R, t)$ as outlined in equation (1) above. This predicted $M(R, t)$ is compared with the data in Fig. 3 inset. Again the prediction is in good agreement with the data.

Several effects modify the simplified theory outlined above. Non-circular drops must have uneven deposition rates: highly convex regions have a stronger evaporating flux and thus denser deposits, as corroborated by Fig. 1a. If the solute is not dilute, the ring deposit is forced to have a non-zero width; higher initial concentration leads to a wider ring. Further thermodynamic effects may modify the flow and the ring deposition. Some solutes may segregate to the substrate surface and become immobilized. Others may segregate to the free surface where the outward flow is faster than $\bar{v}$. The thermal and concentration gradients caused by evaporation can lead to circulating flows driven by surface-tension gradients (that is, Marangoni flows). These can interfere with the outward flow discussed above. Our experiments showed both surface segregation and circulating flow but their effect was minor (as we will discuss in detail elsewhere). High viscosity in the liquid can also modify the deposition by preventing the drop from attaining an equilibrium droplet shape. We estimate that in our experiments such viscous effects should be negligible except within a few micrometres on the contact line.

Our measurements support this capillary flow mechanism for contact-line deposition. They show that the deposition can be predicted and controlled without knowing the chemical nature of the liquid, solute or substrate. The model accounts in a natural way for the nearly complete transport of the solute to the periphery: it also predicts that we can control the shape and thickness of the deposit by controlling the speed and spatial variation of the

evaporation. Often it is desired to deposit solute particles in a confined region as, for example, in the printing of fine lines⁵. The commonplace ring stain seems to provide a simple and robust route to such a goal. □

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Polymerized colloidal crystal hydrogel films as intelligent chemical sensing materials

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Chemical sensors¹ respond to the presence of a specific analyte in a variety of ways. One of the most convenient is a change in optical properties, and in particular a visually perceptible colour change. Here we report the preparation of a material that changes colour in response to a chemical signal by means of a change in diffraction (rather than absorption) properties. Our material is a crystalline colloidal array^{2–12} of polymer spheres (roughly 100 nm diameter) polymerized within a hydrogel^{13,14} that swells and shrinks reversibly in the presence of certain analytes (here metal ions and glucose). The crystalline colloidal array diffracts light at (visible) wavelengths determined by the lattice spacing^{2–12}, which gives rise to an intense colour. The hydrogel contains either a molecular-recognition group that binds the analyte selectively (crown ethers for metal ions), or a molecular-recognition agent that reacts with the analyte selectively. These recognition events cause the gel to swell owing to an increased osmotic pressure, which increases the mean separation between the colloidal spheres and so shifts the Bragg peak of the diffracted light to longer wavelengths. We anticipate that this strategy can be used to prepare ‘intelligent’ materials responsive to a wide range of analytes, including viruses.

Many polymer hydrogels change volume in response to marked changes in environmental conditions, such as temperature, solvent and pH^{15–27}. For certain gel characteristics, these volume changes can be abrupt (volume transitions) as the control parameter is varied. To use such volume changes for chemical sensing, functional groups can be attached to the polymer chains that interact selec-

tively with a single solute species. Such intelligent hydrogels might be used in chemomechanical systems^{28–30} and separation devices^{31–33} as well as sensors^{34–36}. We have recently exploited this kind of response in a temperature-switchable optical diffraction device consisting of a crystalline colloidal array (CCA) polymerized within a hydrogel that responds to temperature changes¹³.

Our sensor materials use a three-dimensional periodic CCA of highly charged polystyrene spheres ~100 nm in diameter to report on the gel volume. Because of electrostatic interactions the spheres self-assemble into a body-centred or face-centred cubic (BCC or FCC) array with a mesoscale periodicity (roughly 100–1,000 nm); thus, the CCA Bragg-diffracts visible light^{2–5}. The diffraction is in the dynamical diffraction regime, and almost⁵ follows Bragg's law:

$$m\lambda = 2nd \sin \theta$$

where m is the diffraction order, λ is the wavelength of light in vacuum, n is the refractive index of the system, d is the diffracting plane spacing, and θ is the Bragg glancing angle.

We fabricate polymerized crystalline colloidal arrays (PCCA) by dissolving non-ionic polymerizable monomers within the CCA suspension and photopolymerizing these species into a hydrogel which entraps the CCA lattice¹⁴. Thus, the CCA lattice spacing, and hence the diffracted wavelength, depends on the hydrogel volume. A change of 0.5% in the hydrogel volume shifts the diffraction by ~1 nm.

We fabricated an intelligent PCCA (IPCCA) sensitive to Pb^{2+} , Ba^{2+} and K^+ by copolymerizing 4-acryloylaminobenzo-18-crown-6 (AAB18C6) into the PCCA. This crown ether selectively complexes Pb^{2+} , Ba^{2+} and K^+ (refs 37–39). Crown ether binding of these particular cations localizes charges onto the gel network (Fig. 1). The gel swelling mainly results from an increased osmotic pressure within the gel due to a Donnan potential arising from mobile

counterions to the crown ether bound cations^{21,27,40}. The cation binding forms a polyelectrolyte hydrogel, whose charge state is determined only by the number of cations bound; the degree of swelling of polyelectrolyte gels increases with the number of covalently attached charged groups^{21,27,40}.

Figure 2 shows that the IPCCA volume and diffraction wavelength monotonically increase with increasing Pb^{2+} concentrations between 0.1 μM (~20 p.p.b.) and 10 mM (~2,000 p.p.m.), at moderate ionic strengths of non-interfering electrolytes (<1 mM LiCl, for example); the shift in the IPCCA diffraction induced by 20 μM $\text{Pb}(\text{CH}_3\text{COO})_2$ (4 p.p.m.) is easily visible to the naked eye. The hydrogel volume maximum occurs at ~10 mM Pb^{2+} where the crown ethers become saturated. At higher Pb^{2+} concentrations the gel begins to shrink because of the decrease in the Donnan osmotic pressure at higher ionic strengths^{21,27,40}. The swelling is reversible; the diffraction reverts to its original wavelength when Pb^{2+} is exchanged out by soaking for a few minutes in de-ionized water. The diffraction peak maxima of the PCCA were reproducible to within 1–2 nm over 200 successive washings and re-immersions in Pb^{2+} solutions.

The selectivity of the IPCCA sensor volume response is determined by the selectivity of the incorporated molecular recognition agent. For example, the 18-crown-6 complex shows log K values (K is the equilibrium binding constant) of 0.8, 2.03, 3.87 and 4.27 for Na^+ , K^+ , Ba^{2+} and Pb^{2+} respectively^{37–39}. Thus, Ba^{2+} swells the PCCA almost identically to Pb^{2+} , making it the major interfering species; K^+ competes with Pb^{2+} for binding only for K^+ concentrations ~200 times that of the Pb^{2+} , and 2,000-fold excess Na^+ is required to compete with Pb^{2+} binding.

Ions that are not complexed by the crown ether only interfere with the IPCCA Pb^{2+} response at relatively high concentrations, because of the impact of their ionic strength on decreasing the

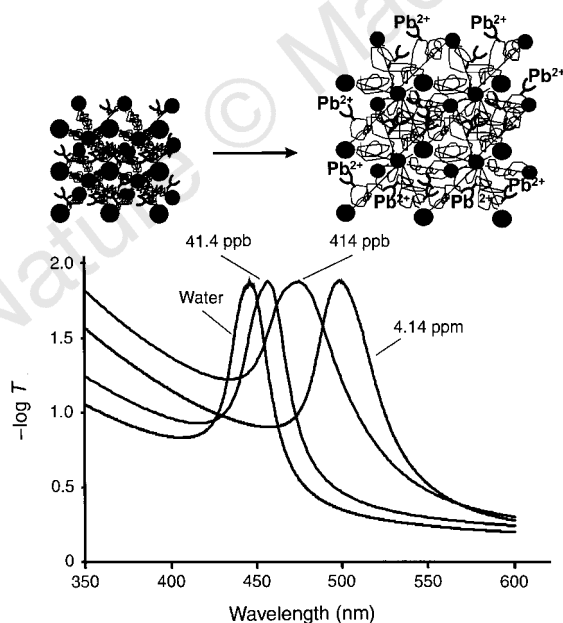


Figure 1 Visible extinction spectra of an acrylamide IPCCA Pb^{2+} sensor at various concentrations of $\text{Pb}(\text{CH}_3\text{COO})_2$. The ordinate is given as $-\log T$, where T is the transmittance. The extinction spectra were measured at normal incidence during immersion of the IPCCA in different Pb^{2+} solutions by using a Perkin-Elmer Lambda-9 ultraviolet-visible spectrophotometer. The IPCCA consists of ~7 wt% polystyrene particles, and ~5 wt% total of polymerizable monomer, cross-linker and crown ether. The relative composition of polymerizable monomers was: 86 wt% acrylamide (AMD), 17 wt% AAB18C6 and 7 wt% N,N' -methylenebisacrylamide (bis AMD), the cross-linker. There is roughly 1 crown ether per 20 AMD, giving an average separation of ~3 nm for a random distribution of crown ether groups.

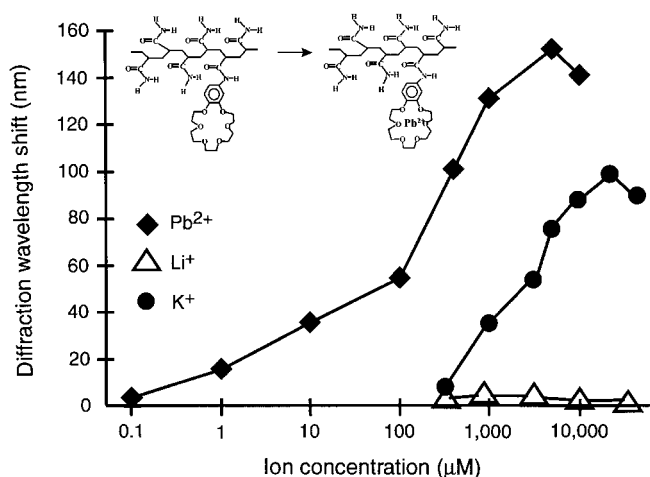
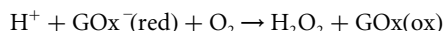
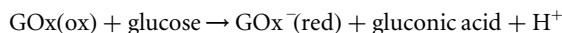


Figure 2 Dependence of the diffracted wavelength of the PCCA sensor on the concentration of cations bound by the crown ether. The cation Pb^{2+} is complexed most strongly, K^+ is more weakly bound, and Li^+ binds negligibly. The diffraction peak maximum redshifts with increasing binding of cations. At very high concentrations of complexed ion, the PCCA contracts slightly, causing a small diffraction blueshift. The detectable concentration range for Pb^{2+} is between 2 μM and 10 mM.

Donnan osmotic pressure. For example 1 mM concentrations of Li^+ and H^+ do not affect the response to $1 \mu\text{M}$ Pb^{2+} . However, concentrations of ~ 200 mM dramatically decrease the response to Pb^{2+} . The ions Fe^{3+} , Cu^{2+} and Cd^{2+} , which interfere with electrochemical determinations of Pb^{2+} in water^{41–43}, have no effect on the response to $1 \mu\text{M}$ Pb^{2+} at $10 \mu\text{M}$ concentrations. But at concentrations of $100 \mu\text{M}$ the volume response to Pb^{2+} decreases, and at 20 mM little response to Pb^{2+} is evident.

We also fabricated an IPCCAs glucose sensor by attaching the enzyme glucose oxidase (GOx) to a PCCA of polystyrene colloids. We hydrolysed the PCCA, biotinylated it, and attached⁴⁴ ~ 20 mg of avidinated GOx cubic centimetre of PCCA, giving a spacing of ~ 25 nm between enzymes. Glucose solutions prepared in air cause the IPCCAs to swell and redshift the diffraction as shown in Fig. 3. No response occurs for similar concentrations of sucrose or mannose, because of the enzyme selectivity. The swelling saturates above 0.5 mM glucose because of the formation of a steady state for the conversion of glucose to gluconic acid, coupled to the reoxidation of the GOx by dissolved oxygen. This IPCCAs returns to its original diffraction wavelength after removal from glucose.

The IPCCAs swelling results from formation of a reduced flavin anion upon glucose turnover. The oxidized flavin is uncharged at neutral pH; however, the reduced flavin is anionic at pH 7 (ref. 45). The reduced flavin is reoxidized by O_2 :



The swelling response to glucose decreases at high ionic strength because of the decrease in the Donnan osmotic pressure.

The concentration of anionic, reduced flavins at steady state depends on the concentrations of both glucose and dissolved

oxygen. In the absence of oxidants, the gel responds even to glucose concentrations of 10^{-12} M; the diffraction is redshifted 8 nm within 30 min if the solution is stirred. At constant glucose concentrations, the number of anionic, reduced flavins depends upon the dissolved oxygen concentration (Fig. 4). Reoxidation of the flavin shrinks the IPCCAs because the number of hydrogel charges is reduced.

We also used the Pb^{2+} IPCCAs as an optrode sensor by attaching a small piece of the IPCCAs to the end of an optical fibre. We coupled light into the fibre and detected the light back-diffracted into the fibre. This optrode worked as a dip probe to monitor low concentrations of Pb^{2+} remotely.

The $125\text{-}\mu\text{m}$ -thick IPCCAs reach equilibrium in seconds to minutes, depending on analyte concentration. The $125\text{-}\mu\text{m}$ -thick Pb^{2+} IPCCAs reach equilibrium in 1 mM Pb^{2+} within 30 s, and the $125\text{-}\mu\text{m}$ -thick glucose IPCCAs reach equilibrium in 0.1 mM glucose within 2 min. The response rate of the Pb^{2+} IPCCAs is limited only by analyte diffusion and the collective diffusion of the hydrogel network⁴⁶, whereas enzyme IPCCAs responses can also be limited by enzyme kinetics. Decreasing the IPCCAs thickness, and the monomer and cross-linker content of the hydrogels, markedly increases the response rate. For example, $10\text{-}\mu\text{m}$ -thick IPCCAs should respond within milliseconds; diffraction from these thin IPCCAs are easily visible to the eye.

The swelling of these IPCCAs results from immobilization of charges on the crown ether or the enzyme-bound flavin. As in all polyelectrolyte gels^{21,27,40} swelling results from the competition between the Donnan osmotic pressure and the elasticity of the gel network²¹. This osmotic pressure increases monotonically with the number of bound charges, but begins to decrease at high ionic strengths²¹. However, the glucose IPCCAs still responds to 0.1 mM glucose in 0.1 mM NaCl, and the Pb^{2+} IPCCAs swells in response to 0.1 mM Pb^{2+} in 50 mM NaCl solutions. We will discuss these

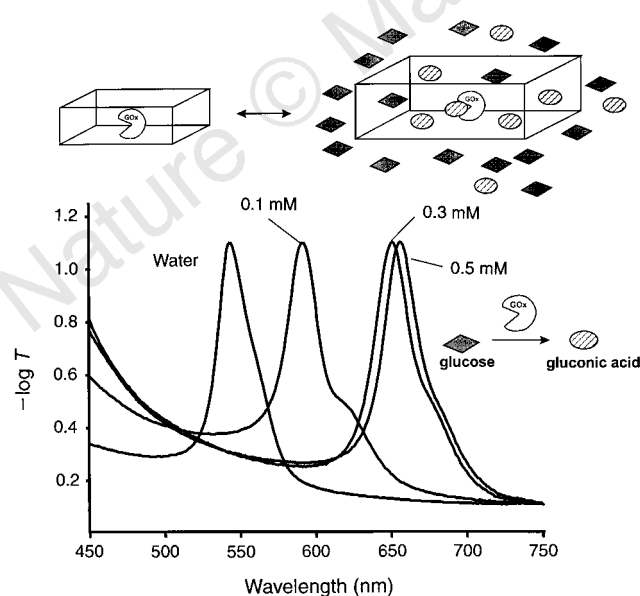


Figure 3 Visible extinction spectra showing how diffraction depends on the glucose concentration for the $125\text{-}\mu\text{m}$ -thick PCCA glucose sensor. The ordinate is given as $-\log T$, where T is the transmittance. The IPCCAs expands for concentrations between 0.1 and 0.5 mM glucose. This IPCCAs was polymerized from a solution containing ~ 7 wt% polystyrene colloidal spheres, 4.6 wt% AMD and 0.4 wt% bisAMD, with water constituting the remaining fraction. This hydrogel was hydrolysed in a solution of NaOH and was then biotinylated with biotinamidopentylamine, which was attached using a water-soluble carbodiimide coupling agent. Avidinated glucose oxidase was then directly added to the PCCA.

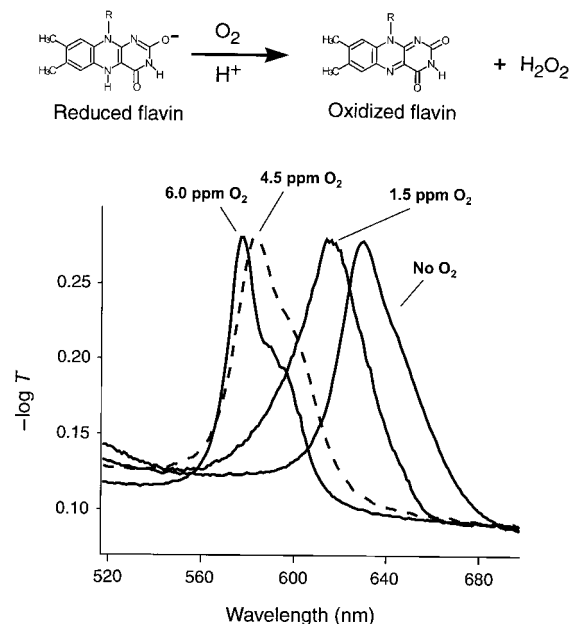


Figure 4 Extinction spectra of the PCCA glucose sensor immersed in an aqueous solution of 0.2 mM glucose with varying O_2 concentrations. The ordinate is given as $-\log T$, where T is the transmittance. The sensor diffracted at 545 nm in the absence of glucose.

phenomena in more detail elsewhere (J. H. H., J. S. W. Holtz, C. H. Munro and S. A. A., manuscript in preparation). Obviously we can tailor the dynamic range of these IPCCAs by controlling the number and affinity of the molecular recognition agents and by altering the elasticity^{21,46–49} of the gel network to define the dynamic range of the sensor response. These sensors could also be used at very high ionic strengths by sample preparation procedures such as dilution or ion exchange.

We can directly quantify the IPCCa response to samples of different ionic strengths by determining the ionic strength using a second polyelectrolyte PCCA gel; we have similarly fabricated a hydrolysed acrylamide PCCA that shrinks in response to ionic strength.

We are also developing IPCCa that do not use bound charges. For example, we can use recognition agents that alter the volume phase transition temperature¹³ as analytes bind to the recognition elements. In addition, we can use recognition agents that alter the free energy of mixing upon analyte binding to alter the IPCCa volume²¹.

Using the same general motif for creating sensor materials and devices but incorporating other crown ethers could produce IPCCAs sensitive to cations other than Pb^{2+} , Ba^{2+} and K^+ . With the use of the avidin–biotin interaction, numerous biomolecular recognition elements could be incorporated into the PCCA. For example, we expect to be able to bind recognition elements such as HIV antibodies in order to fabricate sensors that respond to low virus concentrations.

We expect to use IPCCAs in arrays of optical sensors, where each individual sensor will detect different analytes and different concentration ranges. The diffraction from these IPCCAs is easily visible and can be used for simple colour tests for analytes in solution; the perceived blue diffraction from a $2\text{ }\mu\text{M}$ Pb^{2+} solution shifts to green for a $20\text{ }\mu\text{M}$ Pb^{2+} solution. This technology may prove useful in simple kits for environmental or clinical diagnostics. □

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Solvent-assisted proton transfer in catalysis by zeolite solid acids

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Intermolecular proton transfer in the gas phase is usually strongly disfavoured because the charged products are highly unstable. It is promoted in aqueous solution, however, because the high dielectric constant ($\epsilon = 78.3$) of water allows efficient stabilization of the corresponding cations and anions. Zeolites—microporous catalysts used in petroleum refining and the synthesis of chemical feedstocks—provide another medium for proton-transfer reactions^{1–3}, because their anionic aluminosilicate frameworks are highly acidic. The low dielectric constant of zeolites ($\epsilon \approx 1.6$; ref. 3) suggests, however, that such processes in the zeolite

channels should involve concerted action rather than strong charge separation, and thus resemble gas-phase reactions. Here we demonstrate that solution-like proton-transfer behaviour can be induced in zeolites. We find that the co-injection of nitromethane into a catalytic flow reactor clearly enhances the conversion of methanol, isopropanol and acetone over the zeolite catalyst HZSM-5. Conservation of nitromethane during the course of reaction and its effects on the reactant-zeolite interaction complex seen by solid-state NMR indicate that nitromethane behaves in a manner similar to polar solvents: it promotes proton transfer by stabilizing ion-pair structures. These findings suggest that rationally selected solvents might provide a simple means to increase the efficiency of these industrially important catalysts.

The isotropic ^{13}C chemical shift of acetone-2- ^{13}C , as measured by ^{13}C NMR, is among the most commonly applied spectroscopic measures of relative solid acid strength⁴. The shift is 205 p.p.m. in CDCl_3 solution⁵ and 249 p.p.m. in magic acid solution (an established superacid)^{6,7}. Within the zeolite HZSM-5 used in these investigations ($\text{Si}/\text{Al} = 21$), an intermediate value of 223.7 p.p.m. was measured. Remarkably, we observed significant changes in the acetone-2- ^{13}C shift when various solvents were co-adsorbed at or near saturation coverage. In every case the shift was downfield, suggesting a greater extent of proton transfer (Table 1). The dielectric constants of the solvents generally predict the magnitude of the effect. As control experiments, we repeated the measurements on the molecules listed in Table 1 using the same absolute loadings of acetone and co-adsorbates but a highly siliceous HZSM-5 with a negligible concentration of acid sites. In the absence of acid sites, the acetone chemical shift was independent of co-adsorbate, demonstrating that it is increased proton transfer from the acid site to

acetone, and not a measurement artefact, that accounts for the data in Table 1. Co-adsorbed nitromethane ($\epsilon = 35.9$) produced the largest chemical shift (230.5 p.p.m., $\Delta\delta = 6.8$ p.p.m.) and, thus, was chosen for a more detailed examination of its effect on catalysis.

The ^1H chemical shift of the Brønsted site is strongly sensitive to hydrogen bonding⁸, and the strength of a hydrogen bond is reflected in the magnitude of its shift^{9,10}. Figure 1 shows ^1H magic-angle-spinning (MAS) NMR spectra of zeolite HZSM-5 and various adsorbates measured at 77 K. The 4.3-p.p.m. resonance for the uncomplexed Brønsted site is shifted downfield to 11.3 p.p.m. on complexation with nitromethane (Fig. 1 trace a). This shift is characteristic of normal hydrogen bonds. Complexation with acetone alone (Fig. 1 trace b) results in a chemical shift of 17.4 p.p.m., indicating a stronger interaction. More importantly, the ^1H shift of the hydrogen-bonded complex of zeolite HZSM-5 and acetone is further shifted to 18.6 p.p.m. when the zeolite is saturated with nitromethane (Fig. 1 trace c), providing additional experimental evidence for increased proton transfer with co-adsorption of nitromethane. These observations complement the ^{13}C results in Table 1.

The solvent effect of nitromethane was also reflected in a variety of *in situ* NMR studies in which we observed increased reactivity or formation of charged species. Figure 2 shows ^{13}C solid-state NMR spectra that compare the reactions of dimethyl ether and methylethyl ether on zeolite HY saturated with nitromethane with control experiments lacking co-adsorbed solvent. Without nitromethane, neither ether disproportionated on zeolite HY to form oxonium ions^{11,2}. In contrast, this reaction occurred to a detectable extent at 298 K and to a significant extent at 323 K with co-adsorption of nitromethane. In the case of methylethyl ether, the dimethylethyl-oxonium ion also eliminated ethylene, a reaction originally

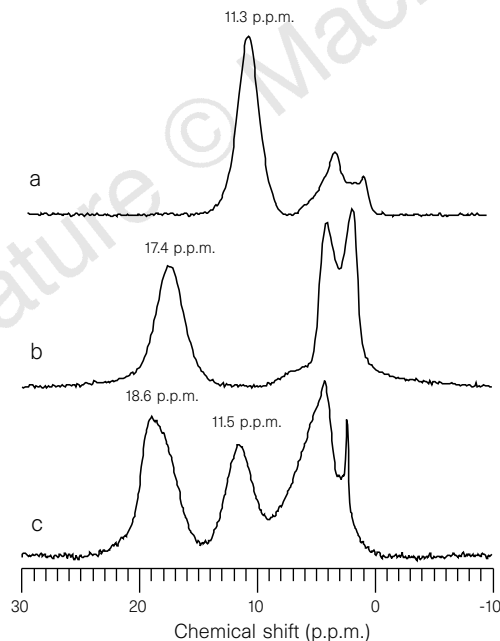


Figure 1 299.6 MHz ^1H MAS spectra probing proton transfer from zeolite HZSM-5 to adsorbates. Adsorptions were done at 298 K on a shallow bed of activated zeolite HZSM-5. Spectra were acquired at 77 K in a 5-mm rotor at a spinning speed of 10,000 Hz. Trace a, nitromethane- d_3 (two molecules per acid site); trace b, acetone- d_6 (one molecule per two acid sites); trace c, acetone- d_6 (one molecule per two acid sites) and nitromethane- d_3 (four molecules per acid site).

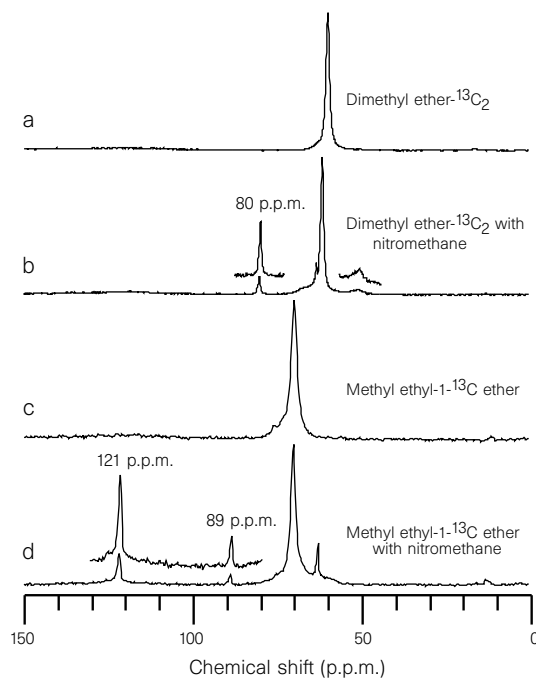


Figure 2 75.4 MHz ^{13}C MAS spectra (Bloch decay) of ethers (one molecule per two acid sites) on ultrastable zeolite HY. Traces b and d are from samples that were saturated with nitromethane at 298 K following the adsorption of dimethyl- $^{13}\text{C}_2$ ether (trace b) or methylethyl- ^{13}C ether (trace d). Spectra were acquired at 327 K with 100 scans and a pulse delay of 8 s. Trace a, unreacted dimethyl ether at 60 p.p.m.; trace b, trimethyloxonium ion at 80 p.p.m.; trace c, unreacted methylethyl- ^{13}C ether at 66 p.p.m.; trace d, dimethylethyl-oxonium ion at 89 p.p.m. and ethylene at 121 p.p.m. The peak at 61 p.p.m. in traces b and d is due to nitromethane. The Si/Al ratio of zeolite HY is 5.5.

proposed¹³ to account for some features of methanol-to-gasoline chemistry on zeolite HZSM-5.

Catalytic flow reactor studies were carried out using He as a carrier gas and pulsed introduction of reactants and solvents. The catalyst was in every case a pelletized form of the same zeolite HZSM-5 used in the NMR studies. We usually delivered premixed reactant and solvent in a single pulse, but we also observed solvent effects when reactant and solvent were introduced onto the reactor in separate, closely spaced pulses. Conversion and product selectivity were determined by analysis (using gas chromatography) of the volatile products, supplemented as necessary by NMR analysis of the organics remaining on the catalyst. ¹³C-labelled nitromethane was used in some experiments to verify that none of the reported products were from solvent reaction or decomposition. Nitromethane was reasonably stable under the conditions used; the only evidence that this solvent formed a product in the zeolite was that a trace of urea accumulated at longer reaction times.

Table 1 ¹³C chemical shifts (δ_{iso}) and chemical shift changes ($\Delta\delta$) of acetone in zeolite HZSM-5 saturated with various co-adsorbates

Co-adsorbate	Dielectric constant	δ_{iso} * (p.p.m.)	$\Delta\delta$ (p.p.m.)
None†	—	223.7	0.0
<i>n</i> -pentane	1.8	224.9	1.2
Bromomethane	9.8	225.9	2.2
1-Nitropropane	23.2	226.3	2.6
Nitroethane	28.1	227.4	3.7
Nitromethane	35.9	230.5	6.8
Acetonitrile	37.5	228.4	4.7

* Dielectric constant indicates the bulk dielectric constant (ϵ) of the co-adsorbates.

† All the chemical shifts were measured from cross-polarization spectra acquired at 298 K. Acetone loadings were less than one molecule for every two acid sites.

‡ The estimated dielectric constant for evacuated zeolites is 1.6 (ref. 3).

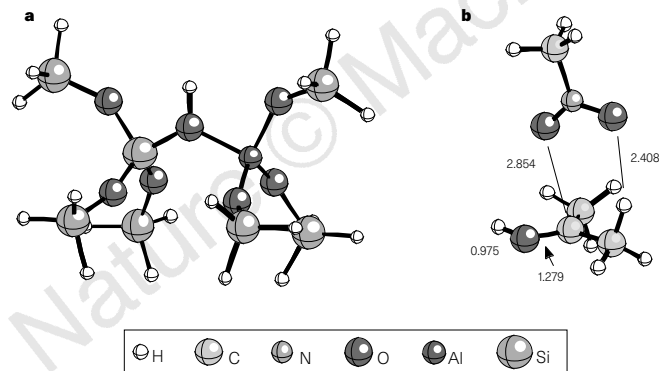


Figure 3 Models used for theoretical calculations. **a**, The HZSM-5 cluster model used to calculate theoretical zeolite acidities as a function of dielectric constant. Optimizations were done on the neutral ($\text{H}_3\text{SiO}_3\text{SiOHAl}(\text{OSiH}_3)_3$) and deprotonated ($\text{H}_3\text{SiO}_3\text{SiOAl}(\text{OSiH}_3)_3$) clusters using density functional theory^{18,19}. The outer shells of the clusters were held fixed in crystallographic positions while the acid site and its first- and second-neighbour shells ($-\text{O}_3\text{SiOHAlO}_3-$ for the neutral cluster) were allowed complete flexibility. Frequency calculations were used to estimate zero-point and vibration contributions to the enthalpy. The optimizations were done using the Slater-Vosko-Wilk-Nussair²⁰ exchange-correlation functional and the DZVP2 basis set²¹. The effects of the dielectric medium were included with single-point RHF/6-31G* calculations using the polarized continuum model²². This model calculates the dielectric stabilization using the van der Waals surface of the molecule (radii of H, 1.2 Å; C, 1.7 Å; O, 1.4 Å; Al, 2.25 Å; Si, 2.1 Å were used). All calculations were done with the program Gaussian94²³. **b**, B3LYP/6-311++G** (level of theory) geometry of the nitromethane-acetone complex in the gas phase after protonation of acetone. Selected distances are shown (Å). The counterpoise-corrected proton affinity of the complex is 209.3 kcal mol⁻¹. Protonation is 16.0 kcal mol⁻¹ more favourable than for acetone alone (B3LYP/6-311++G** = 195.3 kcal mol⁻¹). For comparison, the experimental gas-phase proton affinity of acetone is 196.7 kcal mol⁻¹.

Isopropanol dehydration was studied because it is a commonly applied test reaction for measuring the activity of acidic catalysts. We compared the conversion of solutions composed of 0.20 mole fraction of 2-propanol-2-¹³C with either *n*-pentane or nitromethane as solvents. At a reactor temperature of 433 K, the nitromethane solution produced a yield of 10 times more gas-phase propene than did the *n*-pentane solution. Furthermore, NMR analysis of the fraction remaining on the catalyst revealed much more extensive oligomerization and cracking when nitromethane was present.

Methanol-to-gasoline chemistry was studied as an example of solvent effects in a commercial catalytic process. The effect of nitromethane on the production of hydrocarbons from methanol was so pronounced that we achieved high conversions for fixed contact times at temperatures 50–100 K lower than in otherwise identical experiments without nitromethane.

We also studied the conversion of acetone in the flow reactor to provide a link between the spectroscopic evidence that led us to consider solvent effects and practical catalysis. Acetone dimerizes on acidic zeolites and 'cracks' to form acetic acid and hydrocarbons. Without nitromethane, no detectable conversion to acetic acid was observed at 513 K. When two moles of nitromethane were co-injected with each mole of acetone, 15% conversion to acetic acid was measured at 473 K, and some acetic acid was detected at temperatures as low as 393 K. Analogous results were obtained with *in situ* NMR experiments probing solvent effects on acetone reactivity.

Theoretical calculations confirmed the effect of co-adsorbed solvent molecules on zeolite acidity. The acidity calculated for a zeolite cluster model containing a Brønsted acid site (shown in Fig. 3a) and its deprotonated analogue was found to drop rapidly with inclusion of even a modest dielectric constant: the predicted acidity of the zeolite cluster in vacuum ($\epsilon = 1$) is 285 kcal mol⁻¹, but this value reaches a limiting value of ~250 kcal mol⁻¹ at dielectric constants of 10 or greater. In addition to increasing zeolite acidity, co-adsorbed solvents may also affect the ability of probe molecules to accept protons. Our calculations indicate that the proton affinity of an acetone-nitromethane complex is ~16 kcal mol⁻¹ higher than that of acetone alone. Figure 3b shows the optimized geometry of nitromethane interacting with protonated acetone, illustrating that the relatively long distance (2.85 Å) between C-2 of acetone and the closest oxygen of nitromethane does not prevent significant solvent stabilization effects.

Oligomers of reactants or products might also contribute to acid catalysis in zeolitic reactions, and such reactions could be considered as special cases of solvent-assisted proton transfer. Recent theoretical studies have examined, for example, proton transfer from zeolites to monomers and dimers of methanol and water^{14–17}. In the present investigation, in contrast, we introduced a rationally selected solvent, nitromethane, to enhance proton transfer from the zeolite to the reactant, thereby obviously affecting the kinetics of several catalytic reactions. This solvent effect is purely catalytic in nature (nitromethane is neither a product nor reactant in any of the reactions) and therefore bridges the gap between solution and zeolite chemistry. □

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Prolonged stratospheric ozone loss in the 1995–96 Arctic winter

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It is well established that extensive depletion of ozone, initiated by heterogeneous reactions on polar stratospheric clouds (PSCs) can occur in both the Arctic and Antarctic lower stratosphere^{1–9}. Moreover, it has been shown that ozone loss rates in the Arctic region in recent years reached values comparable to those over the Antarctic^{8,9}. But until now the accumulated ozone losses over the Arctic have been the smaller, mainly because the period of Arctic

ozone loss has not—unlike over the Antarctic—persisted well into springtime^{8–10}. Here we report the occurrence—during the unusually cold 1995–96 Arctic winter—of the highest recorded chemical ozone loss over the Arctic region. Two new kinds of behaviour were observed. First, ozone loss at some altitudes was observed long after the last exposure to PSCs. This continued loss appears to be due to a removal of the nitrogen species that slow down chemical ozone depletion. Second, in another altitude range ozone loss rates decreased while PSCs were still present, apparently because of an early transformation of the ozone-destroying chlorine species into less active chlorinenitrate. The balance between these two counteracting mechanisms is probably a fine one, determined by small differences in wintertime stratospheric temperatures. If the apparent cooling trend in the Arctic stratosphere¹¹ is real, more dramatic ozone losses may occur in the future.

The polar lower stratosphere in winter is characterized by a cyclonic wind circulation (the polar vortex) encompassing a relatively isolated low-temperature region where PSCs can form. The cold surfaces provided by PSCs allow a number of reactions to proceed. The main effect of these reactions is to convert the longer-lived chlorine reservoir species HCl and ClONO₂ into temporary reservoir species such as Cl₂ which are readily photolysed into the species involved in ozone destruction cycles (Cl, ClO, Cl₂O₂ = ClO_x). Fast chemical ozone loss can occur in the presence of sunlight while ClO_x is present^{12,13}. When the temperatures rise so that the PSCs evaporate activation of chlorine is no longer possible. In the Arctic, deactivation occurs primarily through ClONO₂ formation by the reaction of ClO with NO₂, which is produced mainly by the photolysis of HNO₃ or by reaction with OH (refs 14, 15).

Here we report results of the Match 95–96 experiment conducted from mid-January to early April 1996. During this experiment coordinated ozonesonde launches were used to determine chemical ozone loss rates in the Arctic polar vortex.

Trajectory calculations were used to trigger the launches of ~600 ozonesondes in such a way that the ozone concentrations in a large number of air parcels were each measured twice, a few days apart ('matches'). The differences in ozone, when averaged, are interpreted as being caused by chemical processes, as dynamic processes are assumed to be allowed for in the trajectories used. Similar experiments in 1991–92^{4,8} and 1994–95⁹ found significant ozone loss rates during and immediately after (<14 days) periods of PSC existence. A full description of the Match approach can be found in Rex *et al.*⁹ As in the previous experiments^{8,9} the area-weighted sampling of different intervals of potential vorticity inside the vortex with match events was again quite homogeneous. This implies a relative uniform coverage of the vortex air so that the results can be interpreted as vortex averages.

The winter 1995–96 was one of the coldest in the 30-year record of analyses by the Free University of Berlin¹⁶ and the coldest in the 18-year NCEP (National Centers for Environmental Predictions) record¹⁷. Temperatures in the Arctic polar vortex were continuously low enough to form Type I PSCs from early December 1995 to early March 1996. After 11 March, the minimum temperature in the data from the European Centre for Medium Range Weather Forecasts (ECMWF) stayed at least 4 K above the equilibrium temperature of nitric acid trihydrate (used here as indicator for Type I PSC formation; see Rex *et al.*⁸ for details). PSCs were observed by lidars at Ny-Ålesund, Spitsbergen (Norway) and Andøya (Norway) in nearly all measurements between 1 January and 29 February, but no PSC observation was reported during March although measurements continued^{18,19}. The Microwave Limb Sounder (MLS) on the Upper Atmosphere Research Satellite observed a persistent local minimum of gas-phase HNO₃ from the end of January to 3 March, which also indicates that PSCs occurred throughout this period²⁰. The cold temperatures and the

local HNO_3 minimum were located over the Atlantic sector of the polar vortex, stretching from the vortex core to the vortex edge. Owing to the circulation of air masses in the vortex, a large fraction of the vortex air mass passed through this region every few days. During January the temperatures at an altitude of ~ 21 km stayed below the threshold for ice-particle (Type II PSC) formation for 3 weeks, the longest period ever observed in the Arctic¹⁶. The vortex, as defined by large values of potential vorticity and a steep gradient of this quantity at the vortex edge, still existed at the end of March.

Extensive and pervasive photochemical ozone loss occurred over a wide altitude range for several weeks in January–February 1996 (Fig. 1a). In early March the vortex warmed above the PSC threshold and the ozone loss rate dropped rapidly, reaching insignificant values at most altitudes after a few days. The maximum loss rates occurred in January, and these rates are similar to those found during 1991–92^{4,8} and 1994–95⁹. Where comparable (during Feb-

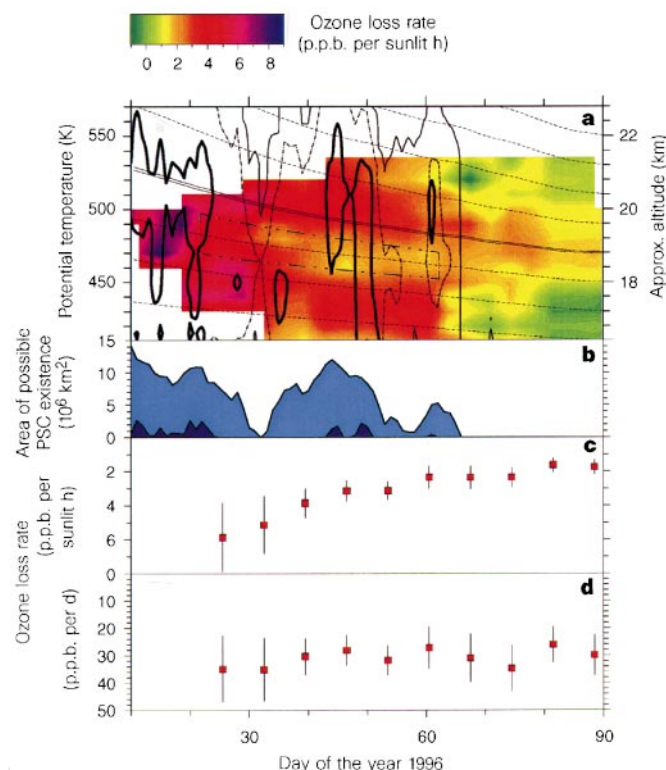


Figure 1 Ozone loss rate and PSC probability in the Arctic atmosphere during January–March 1996. **a**, Contour plot showing the ozone loss rate in p.p.b. per sunlit hour as a function of time and altitude. Potential temperature is used as a measure of altitude. The approximate geometric height of the various potential temperature levels is given on the right-hand scale. 667 matches inside the polar vortex contributed to the plot. The vortex edge was chosen at a scaled potential vorticity of 136 s^{-1} which corresponds to $36 \times 10^{-6} \text{ K m}^2 \text{ kg}^{-1} \text{ s}^{-1}$ in 475 K (ref. 8). The thin contour lines are the $0.3 \times 10^6 \text{ km}^2$ (solid lines) and $4 \times 10^6 \text{ km}^2$ (dash-dotted) isolines of the geographical area covered with temperatures low enough for Type I PSCs to form. The heavy solid contour line is the $0.3 \times 10^6 \text{ km}^2$ isoline of the area covered with temperatures below the ice frost point. The dashed lines correspond to surfaces that follow the diabatic descent of the air masses during the winter. The vertical motion of the air masses inside the vortex was calculated from modelled diabatic cooling/heating rates⁹. See text for the meaning of the dash-dot-dotted box. **b**, **c**, Data as a section along the double line in **a**, reflecting the situation in a subsiding layer of air which started at 535 K potential temperature on 1 January and reached 470 K on 31 March. For this layer **b** shows the geographical area covered with temperatures low enough for the formation of Type I PSCs (light blue) and Type II PSCs (dark blue). **c**, Ozone loss rate in p.p.b. per sunlit hour. **d**, Ozone loss rate in p.p.b. per day. The error bars in **c** and **d** denote the 1σ uncertainty.

ruary on the 465 K potential temperature surface) our loss rates are in good quantitative agreement with those derived from observations of MLS¹⁷. Substantial ozone losses were also derived from observations of the Halogen Occultation Experiment (HALOE)⁷ and from lidar data⁶.

A closer look at Fig. 1a reveals three additional features. (1) Ozone loss persisted throughout March over a limited altitude range

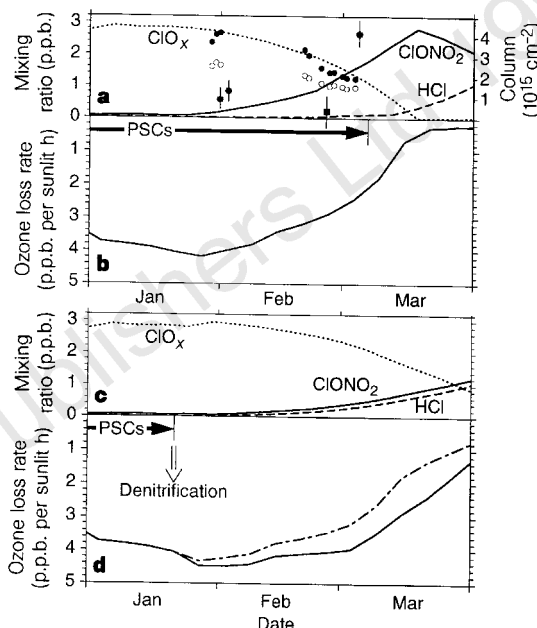


Figure 2 Results from a box model run on an idealized trajectory at 20 km height, oscillating between 60° and 80° N with a period of 6 days. The model was initialized at the beginning of December. **a**, **b**, Results of a model run where 8% PSC probability was assumed between 1 December and 7 March, which led to a total activation of chlorine until 1 January. **a**, Mixing ratios (by volume) of ClONO_2 (solid line), HCl (dashed line), ClO_x (dotted line). The model results are compared with ClO_x vortex averages (filled circles without error bars) calculated from MLS ClO measurements (open circles)²⁰. For this, only ClO measurements at solar zenith angles $< 87^\circ$ were used and only those days are shown when the vortex was sufficiently covered by such measurements. For the calculation of the ratio ClO/ClO_x ECMWF temperatures and reaction constants from De More *et al.*²⁹ were used. The comparison with the simulation shows that the model reproduces the decline of ClO_x inside the vortex surprisingly well. The model shows that the chlorine partitioning is shifted to ClONO_2 although PSCs still exist. This is qualitatively confirmed by ClONO_2 column measurements with a Fourier transform infrared (FTIR) instrument (using the method described in ref. 30) at Ny-Ålesund (78.9° N, 11.9° E)³⁰ even though these measurements represent only a single location. All FTIR measurements (filled circles with error bars) were performed deep inside the polar vortex, and back trajectory calculations show that the probed air masses around 475 K encountered temperatures below the Type I PSC threshold a few days before each measurement. Simultaneous FTIR measurements of the HCl column show that it is constantly very low in early February and early March. The square symbol shows an ASUR observation of the HCl mixing ratio inside the vortex (68.6° N, -6.0° E) at 18 km altitude²⁸. **b**, Ozone destruction per sunlit hour as calculated by the model. The model reproduces the decline of the ozone destruction rate during February, which occurs despite the existence of PSCs, and reveals the underlying mechanism of the 'trapping' of chlorine in ClONO_2 . **c**, **d**, Results (mixing ratios by volume) of a model run where 80% denitrification (for consistency with observations during the slightly warmer winter 1988–89²²) was assumed in late January. No further PSC formation was assumed thereafter owing to a lack of HNO_3 in the denitrified air parcels. In fact, the air parcels in the small denitrified layer inside the vortex and therefore those probed by Match encountered different degrees of denitrification. For example, the dash-dotted line in **d** shows the average ozone loss rate if denitrification is assumed in 50% of the air parcels.

(around the 470 K potential temperature level). This is shown more clearly in Fig. 1b to d. (2) The magnitude of the ozone loss rates generally declined during February by about a factor of two although PSCs were still present. (3) Just below the air in which prolonged ozone loss occurred, a narrow band can be identified (outlined by the dash-dot-dotted box in Fig. 1a) where the decline in the loss rate was more rapid than above and below.

The persistence of ozone loss four weeks after the last PSC occurrence mentioned above (feature (1)) differs completely from the behaviour observed in previous Arctic winters when the ozone loss stopped within ~14 days after the last exposure to PSCs^{4,8,9}. Diabatic cooling calculations (double solid line in Fig. 1a) suggest that in January the air parcels probed in March at around 470 K had been at around 530–550 K. This is the upper part of the region where temperatures were below the threshold for formation of Type II PSCs. Indeed, such clouds were observed at Andøya in three of four measurements during the time period 10–25 January¹⁹, predominantly in the altitude range 525–585 K, and at Sodankylä, Finland, in the altitude ranges 430–454 K and 487–547 K on the 22 January²¹. Vömel *et al.*²¹ have shown that this intensive Type II PSC formation caused a redistribution of water by sedimentation of the ice particles. They concluded that water was permanently removed in a layer with a vertical extent of ~500 m around the 550 K potential temperature level, exactly the air mass in which we later observe the prolonged ozone loss. Although some indications exist that dehydration might take place without denitrification under certain special conditions²², it is accepted that most incidences of dehydration are accompanied by a removal of HNO₃ which is dissolved in the settling Type II PSC particles. Some indications of the removal of gaseous HNO₃ are visible in HNO₃ data measured by MLS²⁰. But because of the limited vertical resolution, any denitrification in a 500-m-thick layer does not appear clearly in the MLS data. The effect of vertical redistribution of water and HNO₃ is common in the Antarctic¹², and measured HNO₃ profiles indicate that it also took place in recent cold winters in the Arctic polar vortex^{22–25}. In a region of severely reduced HNO₃ concentrations the transformation of ClO_x into ClONO₂, and hence the decline of the ozone loss, takes longer as the amount of NO₂ produced by photolysis of HNO₃ is reduced. This effect is illustrated in Fig. 2c and d. We are not aware of any reasonable explanation for the unusual persistence of the ozone loss in the air mass mentioned above other than the assumption that a considerable number of air parcels probed by Match during March around 470 K were denitrified. The observed dehydration in this layer strongly supports this explanation.

A reduction in the magnitude of ozone loss rates in air exposed to Type I PSC for a long time (feature (2) above) has been considered before²⁶, but it has not been observed. Chemical simulations (Fig. 2a and b) show that the first exposure of air to PSCs effectively converts the HCl and the ClONO₂ into ClO_x as they react together on PSC surfaces. In agreement with previous results of similar model studies (see, for example, Müller *et al.*²⁷) any excess HCl reacts on the second or third exposure as, in the interim, ClONO₂ is reformed by the reaction of ClO with NO₂. The simulation suggests that in the extremely cold winter 1995–96 all HCl was consumed. Observations from the Airborne-Submillimeter-SIS-Radiometer (ASUR)²⁸ (Fig. 2a) and from HALOE⁷ support this. Once all the initial HCl has reacted, the fast Cl activation by the reaction of HCl with ClONO₂ is impossible. The most efficient alternative seems to be the reaction of ClONO₂ with H₂O on cold sulphuric acid aerosol (or Type II PSC) surfaces. The reaction rate is strongly temperature dependent, and the sticking coefficients are not well determined (see references in DeMore *et al.*²⁹). The observed decline of the ozone loss rates during February supports the idea that it was hard to recycle ClONO₂ back to ClO_x during this phase of the winter although Type I PSCs persisted. The decline in the ozone loss rate coincides with a decrease of vortex-averaged ClO_x (Fig. 2a) and a strong increase

of the ClONO₂ column inside the polar vortex (Fig. 2a). All observations and the simulation suggest that at the end of February a significant part of the chlorine was trapped in ClONO₂ and could not be recycled by the persisting PSCs.

But in Fig. 1a there is a hint that the ozone loss temporarily accelerates after the second period of possible Type II PSC exposure in the second half of February (in the lower part of the dash-dot-dotted box), consistent with an acceleration in the reaction of ClONO₂ + H₂O due to the colder temperatures.

The more rapid decrease of the ozone loss rate during late January and February mentioned above (feature (3)) occurred in the air mass which was at the base of the Type II PSC region in January (around 490 K), so it is reasonable to suggest that falling Type II PSC particles would have evaporated here. This would enhance the HNO₃ concentrations in this layer. As soon as the Sun shines on this region again, this would lead to enhanced NO₂ concentrations formed by the photolysis of the additional HNO₃. The faster deceleration of the ozone loss rate in this air mass could therefore be caused by an acceleration of the trapping of chlorine in ClONO₂ in this air mass. Because no direct observations of the involved evaporation processes are available this suggestion is in no way proven.

Our results show that prolonged ozone loss, presumably due to denitrification, occurred long after the last exposure to PSCs in the 1995–96 Arctic winter. Features consistent with renitrification of air below this region were found. The cumulative ozone loss in the air mass with prolonged ozone loss reached 2.4 ± 0.3 p.p.m. (~64%) between 20 January and 9 April 1996, the largest loss ever observed in the Arctic. The earlier termination of the ozone loss in the Arctic spring compared to the Antarctic spring (see, for example, refs 8, 10) has been the main difference between the Northern and Southern Hemispheres in the past, and the prolongation of the loss in a limited height region in the 1995–96 Arctic winter is one more step toward the formation of an Arctic ozone hole. However, the spatial non-uniformity of the presented ozone loss rates show also that the balance between the different mechanisms determining the ozone loss rates in the Arctic spring is a fine one, and depends rather sensitively on small differences in the temperature history of the air masses. Predictions of ozone losses in a particular future winter are, therefore, not possible at present. Larger ozone losses over the Arctic could be expected in future, if the apparent cooling trend in the Arctic stratospheric temperatures¹¹ is real. □

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Timing of the Ethiopian flood basalt event and implications for plume birth and global change

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Continental flood basalts are often considered as fossil evidence of mantle plume heads impinging on the lithosphere^{1,2} and have been related to continental breakup^{3–5}. Many of these flood basalts erupted within a short time span—of the order of 1 Myr—and were apparently synchronous with crises in global climate and with mass extinctions⁶. Here we present geochronological (⁴⁰Ar/³⁹Ar) and magnetostratigraphic results for the Ethiopian traps, one of the last remaining flood basalts for which few such data were available. The bulk of the traps, which have been inferred to mark the appearance of the Ethiopian-Afar plume head at the Earth's surface, erupted approximately 30 Myr ago, over a period of 1 Myr or less. This was about the time of a change to a colder and drier global climate, a major continental ice-sheet advance in Antarctica, the largest Tertiary sea-level drop and significant extinctions.

The Ethiopian traps occur near the triple junction of the Red Sea, Gulf of Aden and East African rifts, and have long been associated with the Afar hotspot^{2,7} (Fig. 1). Most of the province lies over the African plate where our study was concentrated. Prolific outpourings of (mainly) basalts have built a subaerial pile which originally covered an area in excess of 500,000 km² (ref. 8), with total thickness locally exceeding 2,000 m. The northwestern traps consist of a series of Late Eocene and Oligocene fissure basalts, covered by Miocene shield volcanoes. Conventional K/Ar ages measured in basalts, rhyolites and ignimbrites from the plateau to the north of Addis Ababa range from⁹ 14 to 40 Myr (Fig. 2). Berhe *et al.*¹⁰ have distinguished three prolonged stages of volcanism at 50–40, 40–30 and 30–21 Myr ago, whereas Ebinger *et al.*¹¹ have proposed that the main phase of volcanism occurred between 45 and 35 Myr ago in the southern part of the Ethiopian rift.

We have investigated three sections of the province. The main section, called the Lima–Limo (LL) section, consists of 2,000 m of lava (Fig. 3) and is located 450 km to the north of Addis Ababa (Fig. 1). A lower 900-m-thick sequence of basaltic lavas ends with 150 m of differentiated products, forming a significant regional terrace level. A second 1,000-m-thick section follows, with an acidic tuff forming another erosional surface at the top of the plateau. This morphology indicates two main volcanic episodes separated by a probable period of quiescence. More recent shield-building volcanism with much smaller volumes ends the LL section. The second section comprises 500 m of the top of the basaltic pile and a capping of ignimbrites and rhyolites at Wegel Tena (WT), 200 km to the north of Addis Ababa (Fig. 1). Another 100 km further north is the third section along the Woreta-Waldia or 'Chinese Road'. The petrology and geochemistry of the sampled formations are described in ref. 12. Samples (50) of whole rocks and plagioclase bulk samples from the basalts, and bulk samples and single grains of feldspars from ignimbrites, rhyolites and tuffs were analysed by the ⁴⁰Ar/³⁹Ar step-heating or total fusion method. A magnetostratigraphy was performed on two of the sections (LL and WT). A more complete description is given elsewhere¹³.

Because the basalts were very fine-grained, it was often not possible to obtain mineral separates, and whole rocks had to be analysed for dating purposes; whenever possible, these were constrained by mineral analyses. The procedure is described and the

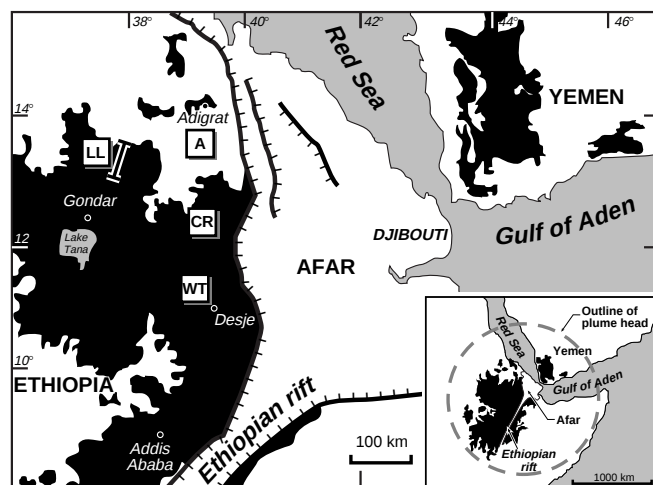


Figure 1 Location map of the northern Ethiopia and Yemen (Oligocene) volcanic province and East African triple junction (after ref. 9, 38). The sections discussed in the text are Wegel Tena (WT), Lima-Limo (LL), Adigrat (A) and 'Chinese Road' (CR).

results are reported in Table 1. Several age spectra are shown in Fig. 4. The spectra provide some evidence for potential sources of disturbance. Isochron calculations were also performed: the best-fit ages were within analytical uncertainty of the plateau ages and yielded atmospheric argon ratios. In the lowermost part of the LL section, a plateau age of 30.8 ± 0.4 Myr was obtained. At intermediate-to-higher levels of the LL section, four plateau ages range from 28.6 ± 0.3 to 30.0 ± 0.3 Myr. Sample E192 was collected from a tuff on the plateau, 50 km away from the main LL section. Step-heating of a bulk feldspar from this sample yielded a plateau age of 30.6 ± 0.1 Myr and an isochron age of 30.1 ± 0.2 Myr. The lowermost lava flow overlying the Jurassic sediments, 150 km away from the LL section at the base of the Adigrat section (A in Fig. 1), yielded a consistent plateau age of 30.4 ± 0.4 Myr. The lowest rhyolite at WT overlies the entire basaltic succession. Three sanidine single grains displayed concordant, high-temperature apparent ages with a weighted mean of 30.2 ± 0.1 Myr. Three sanidine single grains from an intermediate rhyolite, 200 m above the previous sample, also gave concordant high-temperature apparent ages, with a significantly younger mean of 28.2 ± 0.1 Myr. Basaltic flows from the Chinese Road section are capped by rhyolites, analogous to the WT section. Bulk samples of sanidines from two distinct ignimbrites had concordant plateau ages at 29.6 ± 0.1 and 29.4 ± 0.1 Myr. Sanidine single grains gave weighted mean ages concordant with the plateau ages (Table 1).

The histogram constructed from the new argon data shows a sharp single peak centred on 30 Myr with a total width at mid-height of the order of 1.5 Myr (Fig. 2). Comparison of the new Ar/Ar age histogram with the old K/Ar ages reveals the same behaviour that was first found in the Deccan traps^{14–16}. The spread in the K/Ar ages is probably due to alteration rather than actual duration of volcanism. Plateau ages (and one isochron age) from the LL section, together with their uncertainties, are shown in Fig. 3: all but one are between 29 and 31 Myr and not strictly in stratigraphic order. The best estimate for the age of the lower part of the traps is given by the concordant plateau ages of E84 and E216, obtained on whole rocks from different regions (LL and Adigrat). The age of the top of the traps is well constrained by the isochron age of E192 (50 km away from LL), concordant with the plateau ages of the basaltic upper part of LL (PM26 and E100), and the plateau ages obtained on

sanidines from other localities (PM35, E41, E34). Ages for the whole series mostly range from 30.8 ± 0.4 to 29.4 ± 0.1 Myr. A more selective approach consists in selecting only mineral separates which yield consistent plateau and isochron ages, with experimental uncertainties < 1 Myr. This yields a subset of eight ages, which average 29.6 Myr. All of the above argue for the fact that most of the lavas were erupted near 29.5–30 Myr, in probably less than 1 Myr. Baker *et al.*¹⁷ have recently published $^{40}\text{Ar}/^{39}\text{Ar}$ ages from the Yemen traps ranging from 29.2 to 30.9 Myr, in agreement with our values. Somewhat younger ages, corresponding to volumetrically small volcanism and post-dating the main phases of trap emplacement, have also been reported^{17–19}.

Oriented cores were drilled for palaeomagnetic study from 40 flows (30 in LL, 10 in WT), with eight cores taken from each flow.

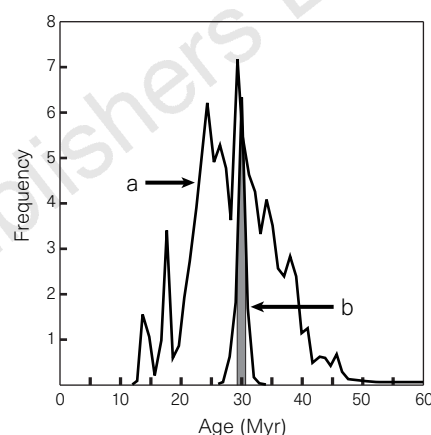


Figure 2 Histograms of Ethiopian volcanic ages. Trace a, 100 K/Ar ages obtained from Ethiopian trap basalts and rhyolites^{9,13,39}, recalculated with the decay constants recommended by Steiger and Jaeger³⁷. Trace b, 10 $^{40}\text{Ar}/^{39}\text{Ar}$ plateau ages (plus one isochron age for E192) reported in this Letter. They include the lowermost trap basalts and the uppermost differentiated formations (rhyolite, ignimbrite, tuff) from the plateau. The shaded area is bounded by the ages at half-amplitude of the $^{40}\text{Ar}/^{39}\text{Ar}$ peak. Each datum is given unit weight and represented by a gaussian distribution with standard deviation equal to the age uncertainty.

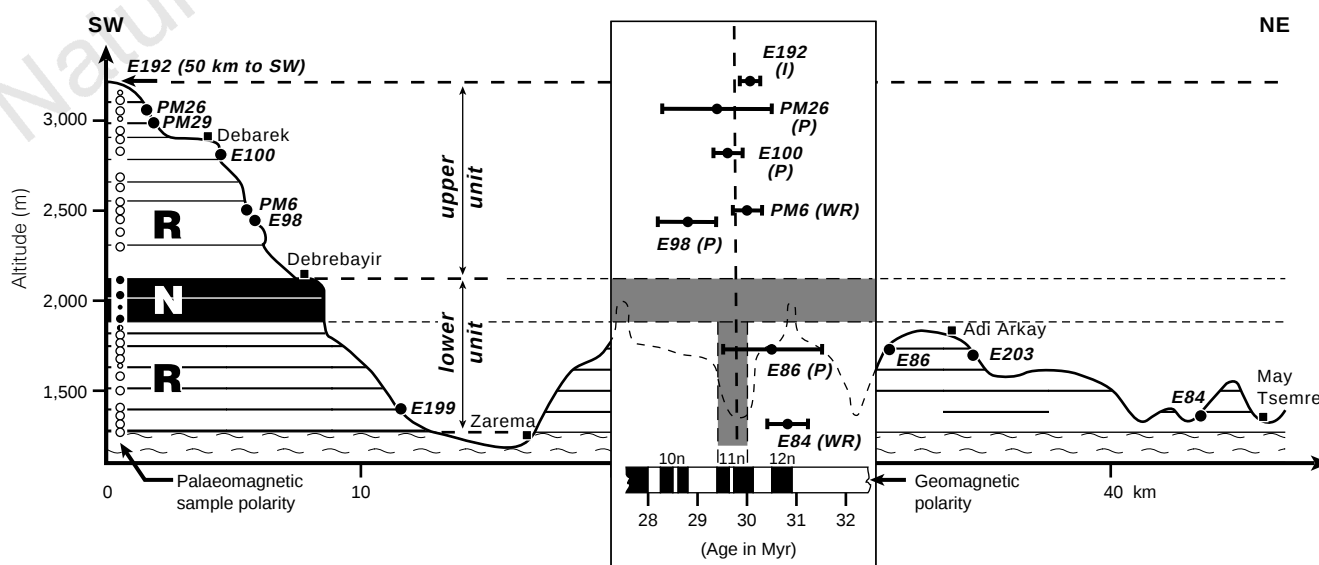


Figure 3 Details of the Lima-Limo (LL) section. Shown are the locations of the samples analysed by $^{40}\text{Ar}/^{39}\text{Ar}$ (black dots along section with sample identification) and magnetic polarity (location and polarity of samples indicated vertically to the left, black for normal polarity, white for reversed polarity). Also shown are the reversed(R)-normal(N)-reversed(R) polarity sequence, the main morphological

breaks defining two volcanic units, the $^{40}\text{Ar}/^{39}\text{Ar}$ ages (P for plateau on plagioclase, WR for plateau on whole rock, I for isochron; see also Table 1), and the geomagnetic polarity timescale²². The suggested correlation of the normally magnetized flows with chron C11n is indicated.

Most sites yielded well grouped, consistent results with unambiguous magnetic polarities¹³. The LL section showed a sequence of only three successive magnetic chrons (11 reversed, 5 normal, and 14 reversed flows). Although very short events (cryptochrons) may have been missed²⁰, observation of only 2 reversals in a pile of 2,000 m thickness, at a time when reversal frequency was of the order of 1.5 to 2 reversals per Myr, confirms that the bulk of volcanism was emplaced in a short time. Given the new radiometric ages, comparison with a recent geomagnetic polarity timescale^{21,22} suggests that volcanism began in the upper part of chron C11r and ended before the onset of chron C10n, implying a maximum duration of 1.5 Myr (Fig. 3). We note that the upper normal-to-reversed boundary occurs at the middle erosional level, consistent with the idea that volcanism occurred in two pulses, each possibly only a few hundred thousand years long. The quiescent period, sufficient to develop the erosional surface, therefore also lasted of the order of a few hundred thousand years. This is consistent with the fact that the ratio of normal to reversed flow thickness is much smaller than the ratio of C11n versus C11r + C10r duration: C11n is incomplete and truncated at the top. The age and reversed polarity of the lowermost ignimbrite in the WT section are consistent with the underlying formations being in chron C10r or C11r. The preliminary magnetostratigraphy at WT displays a succession of 4 reversed, 2 normal, and 2 reversed flows. The age and reversed polarity of sample PM35 would place it in chron C9r. Therefore, the two normal flows in WT could correspond either to chron C10n or C11n, with a gap of about 1 Myr somewhere in the series. This would agree with the uppermost acidic flows corresponding to slower and more sporadic volcanism lingering after the main brief phase of basaltic trap emplacement.

The geochronological and palaeomagnetic results reported above suggest that flood basalt eruptions in Ethiopia occurred in two pulses, beginning shortly before 30 Myr in reversed chron C11r and

ending before 29 Myr in reversed chron C10r, possibly lasting <1 Myr (though no less than 0.6 Myr, the estimated duration of chron C11n). The age, duration and location of Ethiopian trap volcanism have potential significance for continental breakup, global climate change and mass extinctions; only the last two are discussed here. Emplacement of the Ethiopian traps has been linked to the extinction event that defines the boundary between the Eocene and Oligocene epochs in the Cenozoic era. Earlier estimates^{6,23–25} of ages of both events placed them at about 35 Myr. More recent radiometric dating and magnetostratigraphy in pelagic sequences of the northeastern Apennines place the Eocene/Oligocene boundary within reversed chron C13r at 33.7 ± 0.5 Myr (refs 22, 26, 27). The new results presented here rule out temporal coincidence with the Ethiopian traps. Prothero²⁸ has argued that there was actually no great Terminal Eocene extinction event, but rather several periods of cooling (and progressive extinction) through the Eocene and Oligocene. It was not until magnetic chron C11n or C10n that a significant glaciation started in Antarctica. The largest sea-level drop in the entire Cenozoic, evidenced by widespread mid-Oligocene unconformities, was until recently thought to have occurred in chron C10n at the Lower/Upper Oligocene boundary (28.5 Myr)²⁹; this has recently been revised to C11r, very near 30.0 Myr (ref. 30). This period is indeed characterized by one of the maxima in the Cenozoic $\delta^{18}\text{O}$ (oxygen isotope) record^{30,31}, corresponding to a significant cooling event, and also by a minimum in the diversity of land mammals^{32,33}. It is tempting to relate the disrupted world climate around 30 Myr ago to the eruption of the Ethiopian traps. Injection of massive, sulphur-rich aerosols and dust in the atmosphere at a time of long-term cooling could have accelerated global cooling and aridity, triggering an important phase of Antarctic ice-sheet advance and sea-level drop. This period of rapid environmental change could have resulted in low diversity and significant extinctions of species. □

Table 1 $^{40}\text{Ar}/^{39}\text{Ar}$ age determinations for samples from four sections of the Ethiopian traps

Sample label	Material	Plateau age (Myr $\pm \sigma$)	Isochron age (Myr $\pm \sigma$)	$(^{40}\text{Ar}/^{36}\text{Ar})_0$ ($\pm \sigma$)	MSWD	N
Lima-Limo section						
E 192	FE	30.6 ± 0.1	30.1 ± 0.2	312 ± 8	0.6	7
	7 SA	$30.8 \pm 0.2(a)$	ND	ND	3.1	7
PM 26	PL	29.4 ± 1.1	29.8 ± 0.6	295.9 ± 1.9	0.2	11
PM 29	WR	30.0 ± 0.1 (52%)	29.8 ± 0.2	316 ± 16	0.5	6
E 100	PL	29.6 ± 0.3	29.5 ± 0.3	300.8 ± 4.1	0.2	7
PM 6	WR	30.0 ± 0.3	30.0 ± 0.3	299.0 ± 6.0	1	7
E 98	WR	28.6 ± 0.3	28.5 ± 0.5	297.0 ± 6.0	1	7
	WR	28.8 ± 0.6	29.7 ± 0.9	292.5 ± 3.0	0.4	9
E199	PL	31.1 ± 0.6 (51%)	31.3 ± 0.7	294.0 ± 0.6	0.2	4
E 86	PL	30.5 ± 1.0	30.0 ± 0.6	295.6 ± 0.6	0.4	12
E 203	WR	30.4 ± 0.4 (32%)	29.3 ± 0.4	370 ± 27	0.01	5
E 84	WR	30.8 ± 0.4	30.0 ± 0.4	308.0 ± 7.0	0.5	9
Adigrat section						
E 216	WR	30.4 ± 0.4	29.8 ± 0.8	302 ± 12	1.3	10
Wegel Tena section						
PM 37	WR	26.7 ± 0.1	26.7 ± 0.3	282.4 ± 26.0	1.9	6
PM 35	SA	28.2 ± 0.1	28.2 ± 0.1	281.8 ± 15.5	0.6	6
	2 SA	$28.2 \pm 0.1^*$	28.2 ± 0.1	275.7 ± 11.0	0.5	6
PM 31	3 SA	$30.2 \pm 0.1^*$	30.2 ± 0.1	290.1 ± 16.0	0.9	11
Chinese Road section						
E 41	SA bulk	29.6 ± 0.1	29.7 ± 0.2	272 ± 24	1.5	16
	SA	29.5 ± 0.2	ND	ND	ND	4
E 34	SA bulk	29.4 ± 0.1	29.4 ± 0.1	293.3 ± 5.0	2.1	8
	4 SA	$29.2 \pm 0.1^*$	29.5 ± 0.1	289.0 ± 1.7	2.4	4

Material analysed: FE, feldspar bulk samples; SA, sanidine single grains; PL, plagioclase; WR, whole rock. $(^{40}\text{Ar}/^{36}\text{Ar})_0$ is the ratio at intercept, MSWD is the mean squared weighted deviate, and N the number of steps used in age determinations. A plateau was accepted if defined by at least 60% of released ^{39}Ar (a few exceptions are indicated by giving (in column 3) the fraction (%) released), at least three consecutive steps, and if the integrated age of the plateau agreed with each individual age of steps forming the plateau within a confidence level of 2σ . Plateau ages with asterisk (*) represents weighted mean of fusion step ages obtained on several (predegassed and individually analysed) single grains. Samples were irradiated for 70 h in the nuclear reactor at McMaster University (Hamilton, Canada). Maximum flux gradient was estimated at 0.5% within overall sample volume. Whole-rock samples and sanidine single grains were step-heated using a continuous coherent 4W argon ion laser, whereas bulk samples of plagioclase and sanidine were step-heated using a radio frequency furnace. Analytical procedures as given in refs 34, 35. The monitor standard, located about every eight samples in the irradiation can, was the Hb3Gr hornblende, with an age of 1,072 Myr, relative to a formal value of 518.9 Myr for the MMhb-1 standard^{33,36}. Ages were calculated using the constants recommended by Steiger and Jäger³⁷. All errors are quoted at the 1σ level and do not include uncertainties on the monitor age. The error on the measured ratio $^{40}\text{Ar}/^{39}\text{Ar}$ of the monitor is included in the calculation of plateau-age error bars. Abundances of five Ar isotopes were measured (36, 37, 38, 39 and 40), and the $^{40}\text{Ar}/^{39}\text{Ar}$ ratio corrected for the presence of atmospheric Ar, and interfering isotopes of Ar produced by irradiation of ^{40}K , ^{42}Ca , ^{40}Ca and Cl. Calculated salt ratios are: $(^{39}\text{Ar}/^{37}\text{Ar})_{\text{Ca}} \times 10^{-4} = 7.06 \pm 4\%$; $(^{36}\text{Ar}/^{37}\text{Ar})_{\text{Ca}} \times 10^{-4} = 2.90 \pm 3\%$; $(^{40}\text{Ar}/^{39}\text{Ar})_{\text{K}} \times 10^{-4} = 3.02 \pm 3\%$.

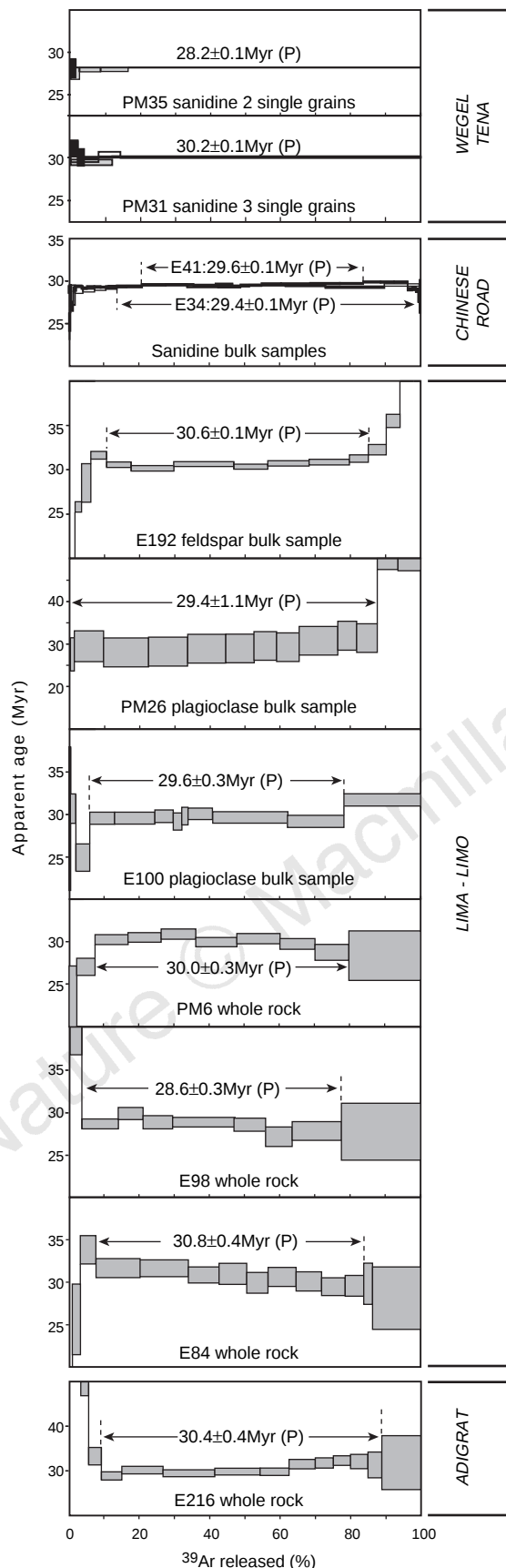


Figure 4 A selection of 11 $^{40}\text{Ar}/^{39}\text{Ar}$ age spectra. Sections are (from top to bottom, see also Table 1) the Wegel Tena, 'Chinese Road', Lima-Limo and Adigrat sections. (P) indicates plateau age.

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Vanadium partitioning and the oxidation state of Archaean komatiite magmas

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The MgO content of komatiite lavas is an important measure of their formation temperature deep in the Archaean mantle, and forms the basis for models of the early Earth's thermal and chemical evolution^{1–5}. Estimates of the primary MgO content of komatiites are sensitive to the oxidation state—characterized by the oxygen fugacity (f_{O_2})—assumed for the magmas during their crystallization. Despite two decades of study, however, f_{O_2} is still poorly constrained for these lavas. Here I present an estimate of the f_{O_2} for komatiite flows, based on the systematics of vanadium partitioning between komatiitic liquid and olivine in six well-characterized komatiite flows of varying ages. This approach shows that the oxidation state of several of these Archaean lava flows was the same as, or possibly more oxidizing than, that of present-day oceanic basalts. These results may require a downward revision of the mantle melting temperature estimated for many komatiites by about 50 °C, and suggest that the mantle was unlikely to be much less oxidized during the Archaean era than at present.

Oxygen fugacity ranges over nine orders of magnitude in natural magmas, showing more variation than any other intensive variable in magmatic systems⁶. Unfortunately, traditional methods for estimating the fugacity of young lavas^{6,7} are not applicable to lavas in most of the geological record because pristine glass or unaltered FeTi oxides are rarely preserved. The f_{O_2} of magmas erupted over time, and their respective mantle source regions, remain to a large degree unconstrained.

Vanadium, like iron, may exist in variable oxidation states in silicate magmas, and crystal–liquid partitioning for this element should be governed by f_{O_2} . Unlike Fe^{2+}/Fe^{3+} ratios, however, the V distribution along well-characterized liquid lines of descent during crystallization may be redox-sensitive, yet remain unaltered during the metamorphism experienced by most lavas in the geological record, such as Archaean komatiites^{8,9}. Vanadium concentrations could thus be a redox indicator, if the distribution of V between melt and liquidus phases were known adequately as functions of temperature, composition and f_{O_2} . Towards this end, the partitioning of V between olivine (ol) and coexisting liquid was determined experimentally (Table 1).

Crystal–liquid partitioning in silicate systems is typically controlled by temperature (T), composition (X) and possibly f_{O_2} . These three variables are often correlated, in some cases preventing rigorous application of the partition coefficients ($D_V^{crystal/liquid}$, where M is any metal cation) to magmatic processes. Results from this study show that $D_V^{ol/liq}$ has essentially no temperature dependence (Fig. 1), and little if any compositional dependence (Table 1), at least over the T – X range of komatiite crystallization. By analogy with solid–solid oxide buffer reactions¹⁰, the lack of a significant temperature dependence may be due to low entropy changes for redox reactions involving V in silicate melts. At a given f_{O_2} , $D_V^{ol/liq}$ is the same (within error) in both komatiite melts (hss15 and komv) having contrasting major element chemistry and levels of V concentration in the melt (0.5 to 1.8 wt%), and in coexisting olivine (0.05 to 0.5 wt%) (Table 1), confirming that Henry's law is obeyed, and that these experiments at enhanced V abundances are applicable to natural systems.

The major variable controlling $D_V^{ol/liq}$ is f_{O_2} (Fig. 1). The reason for the strong f_{O_2} dependence in olivine–liquid partitioning is probably a variable valence state for V in silicate liquids. Preliminary estimates from this and other studies are consistent with V^{3+} and V^{4+} being stable in these melts at terrestrial oxygen fugacities^{11,12} but more spectroscopic information on the valence state of V in silicate melts is required for a complete evaluation.

Depending on the locality, many geochemical studies of Archaean lavas are consistent with V, as well as Zr, Ti, Sc and Yb, being relatively immobile during regional metamorphism, because these elements often show distributions consistent with igneous trends^{8,9,13}. Furthermore, the covariation of V and Zr, Ti, Sc and Yb in individual komatiite flows follows olivine control lines, because olivine is in all cases the dominant liquidus phase in komatiite lavas, having either fractionated from or accumulated in the different zones of solidified komatiite flows^{9,13–15}.

Geochemical data for most komatiite flows show neutral (zero) or slightly negative slopes on plots of Zr/V, Ti/V, Sc/V and Yb/V against V (Fig. 2a–c, e). In four of six flows (Alexo, Kambalda, Saskmar and Morris) this covariation requires that $D_V^{ol/liq} \leq D_{Ti,Zr,Sc,Yb}^{ol/liq}$ during olivine fractionation or accumulation in each flow (see Fig. 2c). Partition coefficients for these elements¹⁶ have the general trend $D_{Sc}^{ol/liq}$ (= 0.15 to 0.4) > $D_{Yb}^{ol/liq}$ (= 0.02 to 0.05) > $D_{Ti}^{ol/liq}$ (= 0.15 to 0.025) > $D_{Zr}^{ol/liq}$ (= 0.001 to 0.01). A very conservative estimate for the Alexo, Kambalda, Saskmar and Morris's flows is that $D_V^{ol/liq} < D_{Ti}^{ol/liq}$ and $\leq D_{Yb}^{ol/liq}$ or, in other words, $0.025 < D_V^{ol/liq} < 0.05$, implying an f_{O_2} of crystallization between $\Delta NNO = 0.3$ to -0.9 , according to the correlation in Fig. 1.

Two komatiite flows from the Barberton Mountain Land, South Africa (Richard's and Stuart's flows, Fig. 2d, f) show different geochemical trends. Richard's flow has variable Zr/V and Ti/V, whereas Stuart's flow shows constant Ti/V but variable Zr/V and Sc/V. Assuming olivine-only fractionation/accumulation, the slopes of these trends in Richard's flow imply $D_V^{ol/liq} > D_{Ti}^{ol/liq}$ (= 0.025) or a maximum f_{O_2} of $NNO + 0.3$, whereas for Stuart's flow, $D_V^{ol/liq} = D_{Ti}^{ol/liq}$ (= 0.025) or $D_V^{ol/liq} > D_{Sc}^{ol/liq}$ (= 0.15)¹⁶, consistent with a range in f_{O_2} between $\Delta NNO = 0.3$ to -3.0 . In the case of both Stuart's and Richard's flows, reduced values of f_{O_2} (or positive slopes of Ti/V, Zr/V and Sc/V against V) are required only if outliers in the geochemical data set are ignored (Fig. 2d, f). There may indeed be geochemical anomalies in these flows due either to analytical problems or to element mobility (see ref. 17).

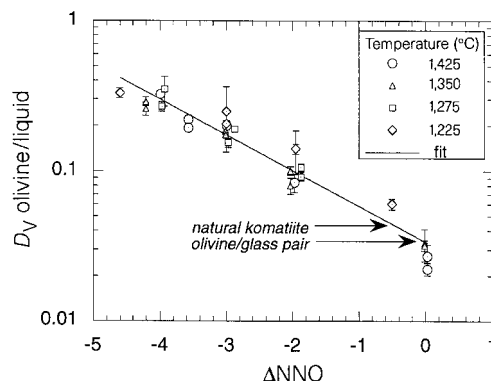


Figure 1 Variation of $D_V^{ol/liq}$ with f_{O_2} (expressed relative to the nickel–nickel oxide oxygen buffer at the temperature of interest (ΔNNO)) for experiments over a range of temperatures. Note that there is little if any temperature dependence of $D_V^{ol/liq}$ (within 1 σ uncertainty, as shown by the error bars) at a given f_{O_2} . The correlation of $D_V^{ol/liq}$ with ΔNNO in both komatiite compositions is fitted by unweighted least squares linear regression as: $D_V^{ol/liq} = 0.03e^{(-0.547 \times \Delta NNO)}$, $r^2 = 0.94$. Also shown is the range of values of $D_V^{ol/liq}$ observed for natural olivine phenocrysts containing glass inclusions in a komatiite flow from the Belingwe Greenstone belt¹⁹.

Minor chromite is observed petrographically as a liquidus phase in most komatiites, but its role in changing V in komatiite lavas with greater than 19 wt% MgO varies depending on the f_{O_2} . For example, $D_{\text{chromite/liq}}^{\text{chromite}}$ varies from ~ 3 at an f_{O_2} of $\Delta\text{NNO} = 0$ to ~ 100 at $\Delta\text{NNO} = -4$ (refs 16, 18), whereas $D_{\text{V/liq}}^{\text{ol}}$ varies from ~ 0.02 to 0.3 over the same f_{O_2} range. Calculations show that under any f_{O_2} condition, even small amounts (2%) of chromite fractionation/accumulation result in highly variable Zr/V, contrary to what is observed in the Alexo, Kambalda, Saskmar and Morris's flows (Fig. 2a–c, e). Chromite has crystallized in these flows but probably has not played a significant role during fractionation/accumulation. In contrast, the same trends for Richard's and Stuart's flows that are consistent with reduced oxygen fugacities ($\Delta\text{NNO} = -3.0$) for

olivine-only modelling can be fitted with 5% chromite fractionation/accumulation under oxidizing conditions ($\Delta\text{NNO} = 0$) (Fig. 2f). At conditions more reducing than $\Delta\text{NNO} = -2.0$, any significant ($>2\%$) fractionation or accumulation of chromite in a komatiite flow results in a vector of steep negative slope on plots of Zr/V against V (Fig. 2c), contrary to what is observed in any komatiite flow. Thus, consideration of chromite during crystallization only serves to raise the f_{O_2} of a flow derived from this method, and its role as a fractionating phase in Stuart's and Richard's flows could change the low f_{O_2} estimated for these flows to higher values similar to the overlying Morris's flow in the very same rock formation.

The experimentally determined uncertainty in $D_{\text{V/liq}}^{\text{ol}}$ of 20% at the

Table 1 Olivine–liquid partitioning data

Sample	Temp (°C)	$\log f_{O_2}$	MgO	Al ₂ O ₃	SiO ₂	CaO	TiO ₂	V ₂ O ₃	Cr ₂ O ₃	MnO	FeO*	NiO	Total	D_V	1σ
hss15			31.51	3.42	46.77	5.67	0.33	0.80	0.34	0.19	11.26	0.27	100.67		
komv			18.95	9.06	46.57	8.96		1.72			13.82		99.1		
27komv gl	1,425	-9.1	19.59	9.73	48.19	9.93	0.04	1.96	0.06	n.d.	9.89	n.d.	99.41	0.19	0.010
27komv ol	1,425	-9.1	48.48	n.d.	40.22	0.27	0.01	0.38	0.02	n.d.	9.30	0.01	98.69		
27hss gl	1,425	-9.1	21.48	5.61	53.24	9.55	0.54	0.86	0.36	0.16	7.49	n.d.	99.30	0.22	0.015
27hss ol	1,425	-9.1	49.63	n.d.	40.71	0.24	0.01	0.19	0.18	0.13	7.32	0.02	98.43		
50hss gl	1,425	-8.5	21.77	5.63	51.62	8.95	0.56	0.85	0.39	0.22	8.11	0.02	98.14	0.20	0.013
50hss ol	1,425	-8.5	52.05	0.03	40.55	0.23	0.02	0.17	0.21	0.16	6.70	0.02	100.15		
26hss gl	1,425	-7.5	20.42	5.69	52.35	9.62	0.56	0.95	0.36	0.16	9.04	0.01	99.26	0.08	0.012
26hss ol	1,425	-7.5	49.39	n.d.	40.33	0.25	n.d.	0.08	0.20	0.13	7.92	0.12	98.43		
25komv gl	1,425	-5.5	20.19	9.12	46.10	9.45	0.02	1.84	n.d.	0.01	12.05	n.d.	98.81	0.03	0.005
1 σ			0.63	0.16	0.89	0.15	0.02	0.10	n.d.	0.01	0.43	n.d.	0.16		
25komv ol	1,425	-5.5	48.60	n.d.	40.13	0.24	n.d.	0.05	0.01	0.01	9.52	0.01	98.56		
1 σ			0.59	n.d.	0.29	0.01	n.d.	0.01	0.01	0.01	0.58	0.01	0.52		
25hss gl	1,425	-5.5	21.56	5.26	51.31	9.02	0.48	0.90	0.32	0.17	9.76	0.05	98.98	0.02	0.002
25hss ol	1,425	-5.5	49.71	n.d.	40.62	0.21	n.d.	0.02	0.12	0.13	8.24	0.26	99.31		
23komv gl	1,350	-10.4	15.97	10.87	49.28	11.30	0.04	2.25	0.01	0.04	9.56	n.d.	99.33	0.29	0.020
23komv ol	1,350	-10.4	47.83	n.d.	39.98	0.29	n.d.	0.65	n.d.	0.01	11.35	0.01	100.11		
23hss gl	1,350	-10.4	17.19	6.68	53.43	11.83	0.65	0.97	0.33	0.22	7.70	n.d.	99.02	0.26	0.026
23hss ol	1,350	-10.4	49.53	n.d.	40.78	0.27	n.d.	0.25	0.12	0.14	8.97	0.03	100.10		
42komv gl	1,350	-9.2	15.78	10.75	47.16	10.31	n.d.	2.05	n.d.	n.d.	12.90	n.d.	98.95	0.17	0.010
42komv ol	1,350	-9.2	47.06	0.15	39.33	0.28	n.d.	0.36	n.d.	n.d.	12.38	n.d.	99.56		
42hss gl	1,350	-9.2	17.43	6.58	52.73	10.46	0.63	0.99	0.42	0.23	9.46	0.02	98.98	0.18	0.009
42hss ol	1,350	-9.2	49.75	0.11	40.00	0.25	0.01	0.18	0.26	0.18	9.21	0.06	100.02		
24komv gl	1,350	-8.2	15.72	10.58	46.32	11.17	0.02	2.09	0.09	0.01	10.24	n.d.	96.27	0.10	0.010
24komv ol	1,350	-8.2	47.80	n.d.	40.59	0.28	n.d.	0.21	0.02	0.01	11.72	0.01	100.63		
24hss gl	1,350	-8.2	15.80	6.67	52.81	11.98	0.69	1.13	0.32	0.19	8.68	0.03	98.40	0.08	0.010
24hss ol	1,350	-8.2	48.48	n.d.	40.42	0.30	0.01	0.09	0.17	0.16	9.67	0.20	99.50		
46komv gl	1,350	-6.2	16.19	10.51	46.79	n.d.	n.d.	2.29	n.d.	n.d.	13.62	n.d.	99.39	0.03	0.001
46komv ol	1,350	-6.2	48.32	0.07	40.00	0.25	n.d.	0.07	n.d.	n.d.	11.53	n.d.	100.24		
46hss gl	1,350	-6.2	17.58	6.19	50.68	9.77	0.58	1.00	0.32	0.22	11.24	0.05	97.81	0.03	0.008
46hss ol	1,350	-6.2	49.41	0.04	39.56	0.24	0.01	0.03	0.16	0.16	9.73	0.31	99.66		
44komv gl	1,275	-10.9	13.15	12.34	49.96	11.64	n.d.	2.01	n.d.	n.d.	10.65	n.d.	99.76	0.35	0.079
44komv ol	1,275	-10.9	47.04	0.26	39.79	0.30	n.d.	0.70	n.d.	n.d.	12.66	n.d.	100.76		
43hss gl	1,275	-9.9	13.60	9.05	50.96	13.57	0.86	1.10	0.31	0.21	8.54	0.03	98.25	0.15	0.011
43hss ol	1,275	-9.9	49.25	0.04	39.17	0.34	0.01	0.17	0.19	0.18	9.89	0.14	99.40		
43komv gl	1,275	-9.8	13.22	12.23	49.63	11.57	n.d.	2.25	n.d.	n.d.	10.60	n.d.	99.50	0.19	0.005
43komv ol	1,275	-9.8	47.55	0.08	40.18	0.28	n.d.	0.43	n.d.	n.d.	12.26	n.d.	100.78		
29komv gl	1,275	-8.8	12.44	12.32	48.98	11.69	n.d.	2.54	n.d.	n.d.	11.55	n.d.	99.51	0.11	0.006
29komv ol	1,275	-8.8	46.63	0.07	39.52	0.27	n.d.	0.27	n.d.	n.d.	13.53	n.d.	100.29		
29hss gl	1,275	-8.8	12.77	9.57	50.22	13.93	0.91	1.10	0.25	0.20	8.59	0.02	97.61	0.09	0.006
29hss ol	1,275	-8.8	48.90	0.03	39.42	0.36	0.01	0.10	0.15	0.19	10.43	0.19	99.79		
22komv gl	1,225	-11.6	9.40	14.28	50.17	13.92	0.01	1.15	0.07	0.03	9.36	n.d.	98.54	0.33	0.025
1 σ			0.09	0.13	0.12	0.13	0.02	0.07	0.03	0.03	0.26	n.d.	0.36		
22komv ol	1,225	-11.6	43.65	n.d.	39.05	0.36	n.d.	0.38	0.04	0.01	16.12	0.01	99.61		
1 σ			0.56	n.d.	0.76	0.03	n.d.	0.02	0.06	0.01	0.16	0.01	1.17		
45hss gl	1,225	-10.5	9.99	11.54	49.87	13.13	1.11	0.91	0.16	0.17	10.06	0.04	97.41	0.21	0.012
45hss ol	1,225	-10.5	45.71	0.06	38.89	0.36	0.01	0.19	0.11	0.18	13.65	0.39	99.56		
45komv gl	1,225	-10.5	10.72	13.28	49.73	12.70	n.d.	1.88	n.d.	n.d.	11.02	n.d.	99.33	0.25	0.116
45komv ol	1,225	-10.5	45.63	0.09	39.56	0.34	n.d.	0.47	n.d.	n.d.	14.71	n.d.	100.80		
20hss gl	1,225	-9.4	8.77	14.49	53.08	13.15	1.31	0.64	0.04	0.15	6.74	0.03	99.03	0.14	0.045
20hss ol	1,225	-9.4	45.46	n.d.	39.73	0.43	0.04	0.09	0.15	0.19	13.28	0.43	99.79		
20komv gl	1,225	-9.4	9.09	14.26	49.67	13.77	0.03	1.78	0.11	0.01	9.75	n.d.	98.56	0.14	0.001
20komv ol	1,225	-9.4	43.67	n.d.	39.39	0.36	0.02	0.25	0.05	0.02	16.80	0.04	100.60		
21komv gl	1,225	-7.5	10.74	12.45	48.07	13.05	n.d.	2.49	0.02	0.03	9.68	n.d.	96.59	0.06	0.005
21komv ol	1,225	-7.5	44.98	n.d.	39.25	0.30	0.01	0.15	0.09	0.01	14.59	0.02	99.40		

Starting compositions were a natural komatiite (hss15) from the Barberton Greenstone belt¹⁶ and a synthetic komatiite (komv), synthesized by mixing reagent grade oxides and fusing the mixture into a pure glass at 1,650 °C. Partitioning experiments were performed at 100 kPa from 1,225 to 1,425 °C and f_{O_2} values from NNO to below the iron–wüstite (IW) buffer in a vertical tube, gas-mixing furnace, using Fe pre-saturated Pt wire loops to avoid Fe loss, and bulk compositions doped with 1 to 2 wt% V₂O₅ to facilitate electron microprobe analysis of the anticipated low V in experimental liquidus olivines. Oxygen fugacity was controlled by CO–CO₂ gas mixture and monitored with a solid zirconia electrolyte cell with variations of ± 5 mV during each experiment, corresponding to $\pm 0.05 \log f_{O_2}$ units. Run durations were 24 to 72 h. Coexisting olivine (ol) and glass (gl) were analysed by electron microprobe with Cameca SX50 and JEOL JXA 8900R instruments using a 1 μ m beam operated at 15 kV, 20 nA. Analytical conditions were 20 s on peaks for all elements except V and Cr in olivine (100 and 60 s, respectively). Ten analyses of both olivine and glass were made in each run product, and averaged. Typical analytical uncertainties (at 1 σ level) are given for two experiments (22komv, 25komv) with high and low V in olivine. n.d.: Not analysed or below detection.

* All iron as FeO.

2σ confidence level (Table 1) results in an uncertainty in derived f_{O_2} of ± 0.5 log units. The main limitation on the approach developed here, therefore, is the scatter in V–Ti, Zr, Sc, Yb covariation in some lava flows. This scatter may represent real post-magmatic redistribution, insufficient analytical precision, lack of detailed sampling of the flow, or inapplicability of the approach developed here. In this regard, it is interesting that the most pristine komatiite flow yet discovered, in the Saskmar drill hole (Fig. 2c), shows the best constrained f_{O_2} by this empirical approach, with $\Delta NNO = 0 \pm 1$. Fresh olivine with 6 p.p.m. V from this locality¹⁹ contains melt (glass) inclusions with 140–160 p.p.m. V, and represents a natural olivine–liquid partition with $D_V^{ol/liq}$ of 0.038–0.043, implying crystallization at $\Delta NNO = 0 \pm 0.5$ (Fig. 1), identical to that derived above for the Saskmar locality, and confirming the robustness of the empirical approach. The fact that a similar f_{O_2} is obtained for the Kambalda and Alexo flows which have been metamorphosed to amphibolite and greenschist grades, respectively (Fig. 2a, b) suggests little if any post-magmatic redistribution, even at the high grades of metamorphism experienced by some flows. Analytical imprecision for V, Zr, Ti, Sc and Yb may also affect the slopes of the plots used in

Fig. 2, and this problem might be quite prevalent in the older geochemical data for the Barberton komatiites (Fig. 2d, e, f). More detailed sampling of some flows would clearly be more desirable, as more rigorous f_{O_2} constraints can be obtained for flows that are well sampled, both geochemically and spatially, such as at Alexo and Kambalda (Fig. 2a, b). Unfortunately, many geochemical studies of komatiites either are not for single flows, or, more critically, provide no data for V. Certainly more detailed geochemical data for individual komatiite flows are needed to test this approach further, but the results for the Saskmar locality are very encouraging.

Most importantly, even within the uncertainty in the natural data, it is evident that the oxygen fugacities of komatiite flows modelled in this study ($\Delta NNO \approx +1$ to -2) are similar to or possibly higher than those of present-day oceanic basalts ($\Delta NNO = -2.0 \pm 1.0$)^{6,20}. This relationship seems independent of age, metamorphic grade or geochemical affinity of the komatiites, as both aluminium-depleted and undepleted types, ranging in age from 2.7 Gyr (Alexo, Kambalda, Saskmar) to 3.5 Gyr old (Morris's) can show values of f_{O_2} near NNO. If the f_{O_2} values recorded by basic magmas represent the f_{O_2} of their mantle source region^{6,21}, then the Archaean mantle source for komatiites is not likely to have been significantly less oxidizing than at present. The oxidation state of the Archaean mantle deduced from komatiite f_{O_2} is contrary to the very reduced f_{O_2} recorded by sulphides included in diamonds formed in a reduced Archaean mantle ($\Delta NNO = -4$ to -5)²². Either these diamonds formed in regions of the mantle unrelated to komatiite generation, or komatiites have suffered significant oxidation during ascent and eruption and possibly degassing.

The new estimates for the f_{O_2} of komatiites presented here permit better constraints on their primary liquidus MgO content. On the basis of their liquidus olivine Fe/Mg ratios, the higher values of f_{O_2} near NNO recognized in this study would require lowering the MgO contents of komatiite liquids by 2 to 3 wt%, implying eruption and mantle melting temperatures about 50 °C lower for these rocks than commonly estimated by this procedure⁴. Whether these mantle melting temperatures are representative of the thermal state of the Archaean mantle²³ or of elevated geotherms in mantle jets or plumes^{4,5} still remains controversial.

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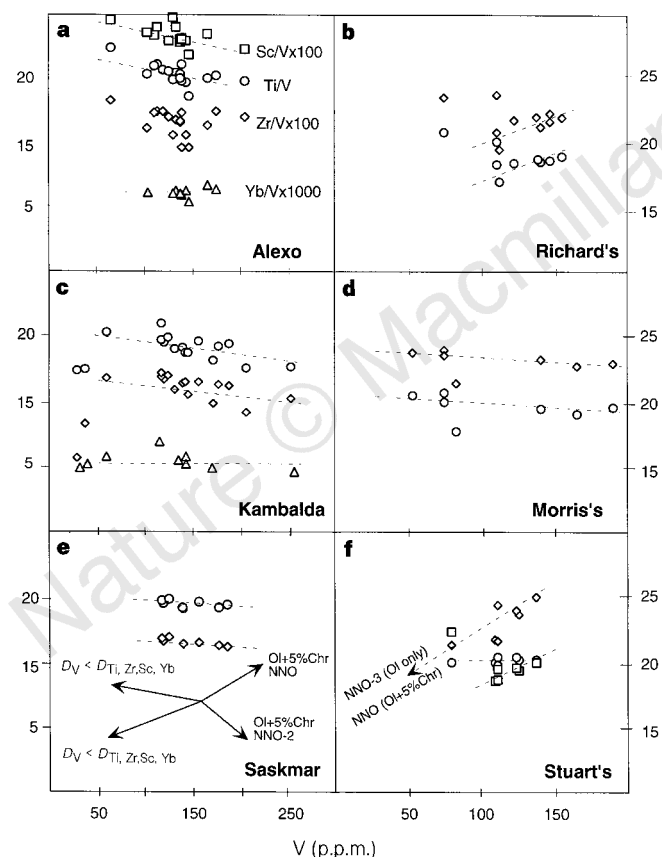


Figure 2 Covariation of V and Ti, Zr, Sc, Yb abundances in six well-characterized komatiite flows. The symbols for each element are given in **a**. The slopes of thin dashed lines fitted to the V–(Ti, Zr, Sc, Yb) covariation in each flow show the relationship between $D_V^{ol/liq}$ and $D_{Ti,Zr,Sc,Yb}^{ol/liq}$ during olivine fractionation/accumulation, as is outlined schematically in **c**. In **f** Rayleigh fractionation/accumulation with 95% olivine and 5% chromite (5% Chr) was modelled using f_{O_2} -dependent $D_V^{chromite/liq}$ data summarized in ref. 18 and compared to chromite-free, 100% olivine (ol-only) trends. Note the outlying data points for trends in the Barberton flows (**d–f**). Data sources are: ref. 24–Saskmar drillhole, Reliance Formation, Belingwe Greenstone Belt, Zimbabwe; refs 25, 26–Morris's, Richard's and Stuart's flows, Komati Formation, Barberton Mountain Land, South Africa; ref. 27–Kambalda flow, Yilgarn Block, Australia; refs 13, 17–Alexo flow, Abitibi Belt, Canada. Note the change of scale from the left to the right hand panels.

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Spatial invariance of visual receptive fields in parietal cortex neurons

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Spatial information is conveyed to the primary visual cortex in retinal coordinates. Movement trajectory programming, however, requires a transformation from this sensory frame of reference into a frame appropriate for the selected part of the body, such as the eye, head or arms^{1–4}. To achieve this transformation, visual information must be combined with information from other sources: for instance, the location of an object of interest can be defined with respect to the observer's head if the position of the eyes in the orbit is known and is added to the object's retinal coordinates. Here we show that in a subdivision of the monkey parietal lobe, the ventral intraparietal area (VIP), the activity of visual neurons is modulated by eye-position signals, as in many other areas of the cortical visual system^{5–10}. We find that indivi-

dual receptive fields of a population of VIP neurons are organized along a continuum, from eye to head coordinates. In the latter case, neurons encode the azimuth and/or elevation of a visual stimulus, independently of the direction in which the eyes are looking, thus representing spatial locations explicitly in at least a head-centred frame of reference.

The occipito-parietal cortical visual pathways contain several anatomically and functionally distinct areas which have been shown in monkeys to participate in specific aspects of spatial analysis and in movement preparation^{11–14}. One of these areas is the ventral intraparietal area. Area VIP receives a major visual projection from the middle temporal area (MT), the principal cortical relay for motion information^{15,16}. Consistent with this input, most of its neurons are sensitive to the direction and speed of moving visual stimuli¹⁷. Although the prevalent sensory modality is visual, VIP neurons are often multimodal, responding to visual, tactile and vestibular stimulation, with parallel preferred directions for all three modalities^{18–20}. The somatic representation emphasizes the face region, and neurons with visual and tactile responses have spatially congruent receptive fields (RFs): that is cells with central visual RFs have tactile RFs located in the middle of the face, and cells with peripheral visual RFs have somatic RFs located on the side of the head. VIP is reciprocally connected to portions of the premotor cortex responsible for head movements, oral prehension and coordinated hand/mouth actions^{21,22,29}. Thus, this area offers an opportunity to investigate multimodal encoding of reference frames and associated coordinate transformations by parietal neurons.

Previous studies that have addressed these issues have found that neurons in most visual areas of the cortex have an RF that remains anchored to the retina^{5–8,10}. There is some evidence, however, that direct encoding of non-retinal coordinate frames exists at the single-cell level. In the lateral intraparietal area (LIP), an area that participates in saccadic eye movements, neurons signal an eye-movement trajectory towards a location in space, even when it is not equivalent to the retinal coordinates of this location^{23,24}. Another study using hand mapping of RF contours described neurons in area PO/V6 responding to a position in space, irrespective of gaze direction²⁵. Finally, in bimodal visual-tactile premotor neurons, visual responses were found to remain anchored to the body part containing the tactile RF, and not to a particular part of the visual field, thus encoding spatial locations in 'body-parts' coordinates²⁶.

We have now investigated the RF of area VIP neurons by using a protocol designed to dissociate a retinal, or eye-centred coordinate system from a head-centred coordinate system. We applied a

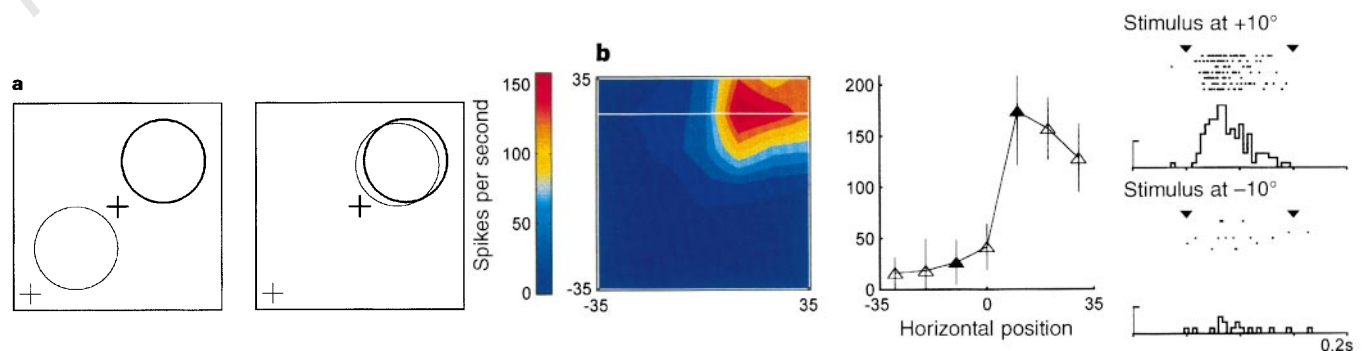


Figure 1 a, Hypothetical retinotopic (left) and spatially invariant (right) receptive fields (RFs). Thin and thick circles correspond to the regions of the screen covered by the RF when the eyes are looking in the direction represented by the matched thin or thick cross, respectively. Left, the RF and the direction of the line of sight are rigidly linked when the eyes move from one position to the other; right, the screen area covered by the RF is invariant, despite the change in eye position. **b**, RF computation. From left to right: colour-coded isofrequency contours of an

actual RF; horizontal slice through the peak of the RF (corresponding to the white line in the contour plot) showing mean firing rate and standard deviation for each stimulus position contained in that slice; rasters of individual trials and time histograms aligned on stimulus onset for a location inside and a location outside the neuron's RF (indicated by filled triangles in the slice plot). Inverted triangles on top of the rasters mark the sampling window used to calculate mean firing rates. (Vertical calibration bar, 200 spikes s⁻¹; tick marks below histogram, 50 ms.)

receptive field mapping procedure that allows rapid stimulation of a large number of locations in the visual field of head-fixed monkeys who were trained to look at one of several possible fixation targets. Different results were expected in this task depending on whether a neuron's RF encoded eye-centred or head-centred coordinates. An eye-centred RF should move rigidly from one fixation location to the next, in the same direction and with the same amplitude as the eyes (Fig. 1a, left). By contrast, a head-centred RF should remain fixed in space despite changes in eye position (Fig. 1a, right). RFs of single neurons were calculated for each fixation location by converting the raw spike trains evoked by each stimulus into a mean firing rate and, after data interpolation, by plotting the results as a colour-coded contour map (Fig. 1b).

We found a wide range of RF types. Some neurons had an RF that moved rigidly with the eyes, whereas other neurons encoded the same location in space irrespective of eye position. The plots in Fig. 2a show, for a single VIP neuron, the distribution of neural activity over the stimulated screen area for nine different eye fixation positions. In each map, the most active region is located in the upper left quadrant of the screen. The fact that the cell's RF remains fixed relative to the stimulation screen indicates that it does

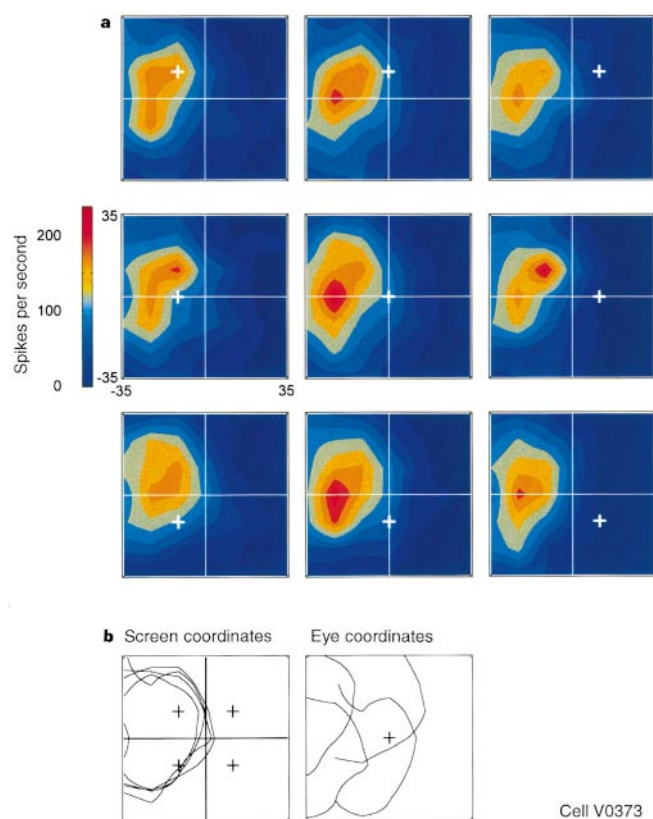


Figure 2 Single-neuron data for visual receptive field mappings in which the RF remains in the same spatial location irrespective of eye position. The RF was mapped with a white bar moving at 100 deg s^{-1} for brief intervals in the neuron's preferred direction. **a**, Colour-coded maps of the RF were constructed for each fixation position. Contour maps represent isofrequency intervals computed from linearly interpolated data matrices. Maps are displayed in screen coordinates. The small white crosses correspond to the eye position during visual stimulation; the intersection of the light horizontal and vertical lines corresponds to the straight-ahead direction in space. **b**, Contour plots for the top left, top right, bottom left and bottom right RF maps show the stimulation region from which firing rates above 50% of peak discharge were obtained. The left graph superimposes the four RF maps as they appear in screen coordinates (as in the colour-coded plots); the right graph represents the same maps but reframed in eye coordinates (relative to the fovea).

not encode a fixed retinal location, but that the retinal locations that drive this neuron vary with eye position. RFs measured at different eye positions spaced 20° apart can be directly compared in Fig. 2b, where contour plots traced at half the maximum firing rate are drawn in both head (screen) and eye coordinates. The best alignment is obtained for RF contours represented in head coordinates. Neurons like the one shown in Fig. 2 exhibited complete compensation for eye displacements, but other neurons compensated partially, sometimes to different degrees for the horizontal and vertical components of eye position, a pattern also observed in a small number of area V6/PO neurons²⁵. Figure 3 shows a neuron whose RF remained fixed relative to the screen when the eyes moved vertically, but shifted partially when the eyes moved horizontally. The upper horizontal borders of the RFs overlap precisely when plotted in screen coordinates (Fig. 3a, left) but appear scattered when plotted in eye coordinates (Fig. 3b, right). The right vertical borders of the RF move in conjunction with the horizontal eye displacements. However, because they do so only by about half of the amplitude of the eye displacement, the vertical borders do not overlap better in eye-centred than in screen coordinates. This neuron therefore encodes the elevation of a stimulus in head-

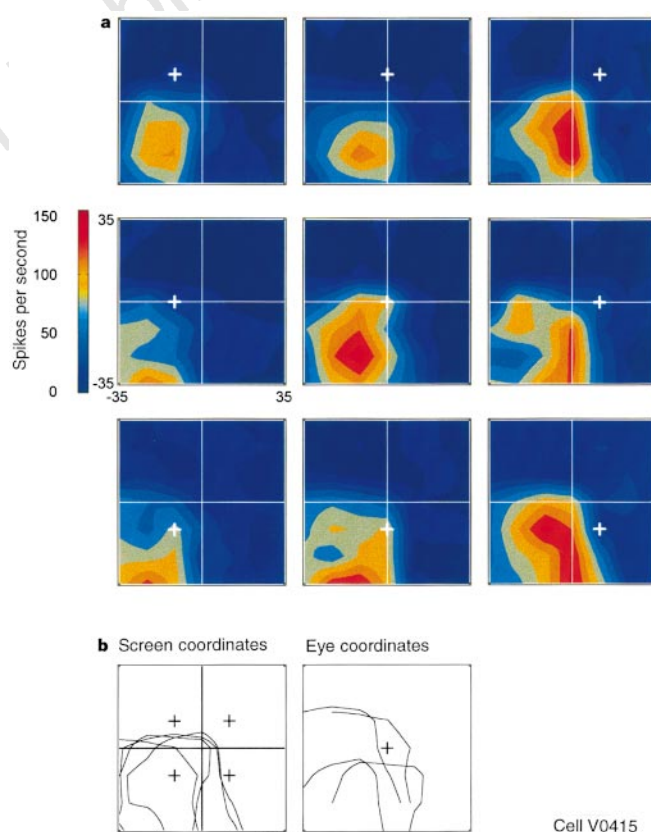


Figure 3 Single neuron encoding elevation in head-centred coordinates. **a**, The upper borders of the RF do not vary with vertical eye position; however the right vertical border is not fixed but is displaced with horizontal changes in eye position. **b**, Contour plots of the RF at the four extreme eye fixations show perfect overlap of the upper RF borders in screen coordinates. The right-side borders do not overlap perfectly in those coordinates, but neither do they when the contours are replotted in eye coordinates. The shift in eye position is thus only partially compensated by a countershift of the retinal response area. Conventions as for Fig. 2.

centred coordinates but it encodes azimuth in an intermediate manner, which is neither fully head-centred nor fully eye-centred.

To quantify the extent to which an RF moves with the eyes, we applied two-dimensional cross-correlation between the nine different maps obtained for the same cell. This procedure allows us to define how much an RF map obtained at a given fixation must be shifted relative to a map obtained at a different position in order to maximize the correlation coefficient. For head-centred RFs, the highest correlation is obtained when the two maps are perfectly superimposed (null shift), whereas for eye-centred RFs, it is obtained when the maps are shifted by an amount equal to the difference in eye position between the two mapping conditions. Thus, the expected ratio of the computed shift to the difference in eye position ($t_{\max}/D_{\text{eye}}$) is 0.00 for head-centred RFs, and 1.00 for eye-centred RFs. For the neuron shown in Fig. 2, the values of the mean shift ratios were 0.04 for vertical and 0.01 for horizontal eye displacements, reflecting a stable position in space of this cell's RF. For the neuron shown in Fig. 3, the shift ratios were 0.09 and 0.55 for vertical and horizontal eye displacements, respectively. The results for 66 VIP neurons are summarized in Fig. 4. There is a continuum between cells whose RF is fixed to the eyes and cells whose RF is fixed to the screen. Between the two extremes were cells whose RF shifted partially or asymmetrically with respect to horizontal and vertical eye positions. We tested whether the distribution of RF shift index was bimodal by using cluster analysis, with a two-means clustering algorithm and horizontal and vertical shift ratios as input variables. This procedure grouped the cells in two normally distributed clusters that were statistically highly different from each other. It seems that although RF shifts as a function of eye position are measured on a continuous scale, the population of VIP neurons studied might actually contain two distinct subpopulations of RF types, linked by an area of gradual transition between retinal and head-centred coordinates.

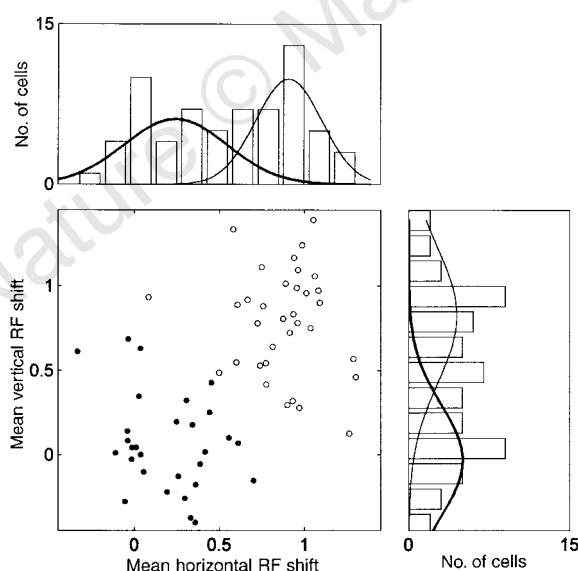


Figure 4 Ratio of horizontal and vertical displacement of the RF to horizontal and vertical displacement of the eyes for all VIP neurons studied. Individual points in the scatter plot represent the mean normalized shift averaged over all possible pairwise comparisons ($m = 36$) of the nine RFs obtained for a given neuron (total: $n = 66$). Open and closed circles correspond to two subpopulations identified through cluster analysis. The mean horizontal and vertical shift indices were 0.20 and 0.00 for the first cluster, and 0.88 and 0.79 for the second cluster (horizontal $F[1,64] = 121.3$; vertical $F[1,64] = 94.7$; both $P < 0.0001$). On top and to the right of the scatterplot are shown the distribution of horizontal and vertical shift ratios, and the normal curve fits to each subpopulation satisfying the Kolmogorov-Smirnov normality test.

As already mentioned, for a cortical area to process a head-centred coordinate frame, retinal and eye position input must be combined. How these two sources of information interact is still unclear. One possibility is that higher-order forms of space representation may be computed from the distributed activity of populations of retinotopic visual neurons whose sensitivity is gated by eye position²⁷. Over half of the neurons (35/66) recorded here showed a significant eye-position effect (at the 0.05 level or better). In most cases (26/35), the effect takes the form of a planar 'gain field' which can be approximated by two-dimensional linear regression. Such eye-position effects occurred across the whole continuum of eye- and head-centred neurons. The cell illustrated in Fig. 3 is a typical example, in which the size of the visually evoked discharge in the centre of the RF increases monotonically as the fixation point moves from the left to the right part of the screen. Several cortical areas, including V3A, MT, MST, PO, 7A, LIP and the premotor cortex, contain neurons whose overall activity is gated by a coarse eye position in a manner similar to our VIP neurons⁵⁻¹⁰. According to the population coding hypothesis, all of these areas carry the necessary signals for generating a distributed head-centred representation of visual space. However, area VIP and probably also area PO/V6 (ref. 25) stand out because their head-centred locations are encoded at the single neuron level. This suggests that space may be represented in the cortex both at the population level and at the single cell level. It is also possible that the eye-position signals that are widely distributed throughout the visual system are used in certain areas to perform spatial transformations while they are merely being passed on by the others.

The functional heterogeneity of the posterior parietal cortex suggests that space is not represented as a single map but instead as a collection of interconnected, specialized areas that play a role in different behaviours^{13,14}. VIP neurons are sensitive to tactile stimuli on the head, to near or approaching visual stimuli, and to vestibular head-motion signals. Although no physiological evidence has as yet linked area VIP to a specific motor behaviour, head-centred coordinates could provide a common reference frame to encode the multisensory signals that converge onto it, and provide targeting and feedback information to premotor areas in the frontal lobes, where neurons describe goals for motor acts using the same coordinate system^{26,28}. The frame of reference encoded by VIP neurons is at least head-centred. However, it is not possible at this point to assert that this represents an end-stage computation. Head-centred RFs may themselves correspond to an intermediate step towards other reference frames. Oculomotor coordinates could be computed before eye movements from a target's head-centred location by subtracting out eye position. Neck proprioception and vestibular signals could also make as as-yet unrecognized contribution. As in our experiments the head was fixed relative to the body and the body fixed relative to the laboratory environment, future studies will have to examine whether individual parietal neurons can provide even higher-order, body-centred or environment-centred reference frames. □

Methods

General. We recorded from three hemispheres of two macaque monkeys (*Macaca fascicularis* and *Macaca mulatta*). Monkey care, training and surgical procedures were carried out in accordance with local and European Community directives. The monkeys were trained to maintain stable fixation on a small spot of light with their heads fixed in return for a liquid reward. Visual stimuli were computer-generated and back-projected by a liquid crystal display system on a translucent screen. Eye position was continuously monitored and the monkeys were required to maintain the eyes within a 2°-wide tolerance window for uninterrupted periods lasting up to 3.5 s. Single unit activity was recorded extracellularly with tungsten microelectrodes. Histological verification of electrode penetrations is not yet available because the animals are still being used for experiments. The location of area VIP within the recording area was identified on the basis of a set of physiological criteria

described previously^{17,18,20}. Briefly, area VIP was found in the fundus of the intraparietal sulcus, at ~7 mm from the cortical surface during electrode penetrations made parallel to the sulcus; the area extends for ~2–3 mm. Access from the lateral bank shows an abrupt transition from non-direction-selective visual and saccade-related activity which characterizes the lateral intraparietal area (LIP), to direction-selective visual responses often accompanied by direction-selective somatosensory response in the face and the head region. Access to VIP from the medial bank of the sulcus through area 5 and the medial intraparietal area is characterized by an abrupt transition from purely hand or arm somatosensory activity, to brisk direction-selective visual and face somatosensory responses.

Receptive field mapping. Each recorded neuron's visual RF was initially located manually and optimal stimulus parameters were identified. The RF was subsequently measured quantitatively while the monkey fixated at one of nine possible locations spaced 10° apart, from 10° up and to the left of the screen centre, to 10° down and to the right. The visual stimulation area covered a 70°-by-70° surface, divided into a virtual square grid of 49 non-overlapping subregions, each one subtending 10° by 10°. A single stimulus consisted of a 10°-by-1° white bar appearing at one edge of a given subregion, moving in the optimal direction perpendicular to the orientation of the bar at a constant velocity of 100 deg s⁻¹ for 100 ms and disappearing, thus covering exactly 10° during a single sweep. The stimulus reappeared at a different location 300 ms later, moved for 100 ms, disappeared, and so on. A stationary 10°-by-10° white square was used when a moving bar did not elicit a reliable response but a flashed stationary stimulus did. Six to eight stimuli were presented in this rapid sequence at randomly selected locations in the course of a single successful fixation trial. The location of the fixation spot also varied randomly from one trial to the next. Nine separate RF maps were thus obtained concurrently during a single experiment. For each fixation location, we recorded a complete stimulation grid with 6–10 stimulus presentations per grid position.

RF maps were constructed off-line by counting the total number of spikes evoked by stimulating a given grid subregion using a shifted temporal window adjusted to the cell's response latency. Spike count was averaged over the number of presentations and converted into mean firing rates. The resulting 7 × 7 raw matrix was subsequently converted into a 25 × 25 matrix by linear interpolation of three new points between each original data point, and plotted as colour-coded contours connecting isofrequency regions. The number of contours in the plot correspond to the discretization of the firing rate, scaled to the maximum firing rate over the nine RF maps obtained for a given neuron. The contour outlines plotted in panels b of Figs 2 and 3 correspond to a line traced around the area for which firing is at least 50% of the peak rate for the corresponding RF map.

Cross-correlation analysis. An estimation of RF displacement in conjunction with changes in eye position was obtained using a cross-correlation analysis. This approach allows us to make use of all the information available in the neural activity maps. It provides a more robust estimate of RF displacement than measures based on peak location or centre of mass, especially when an eccentric portion of the RF exceeds the stimulation area. It is also insensitive to variation in the absolute level of activity associated with the presence of a gain field. In essence, the procedure involves calculating correlation coefficients between two maps (for example, the interpolated activity matrices) that are systematically displaced relative to one another step by step, row-wise and column-wise. The shift associated with the highest correlation coefficient ($t_{\max}$) was normalized with respect to the difference in eye position (D_{eye}), yielding the ratio $t_{\max}/D_{\text{eye}}$. For a neuron strictly encoding eye-centred coordinates, the shift that maximizes the correlation coefficient corresponds to the difference in eye position associated with the maps. For example, when cross-correlating a pair of maps obtained during fixation at 10° to the left and at 10° to the right, $t_{\max}$ is obtained with a pure horizontal 20° shift. As D_{eye} is also equal to 20°, $t_{\max}/D_{\text{eye}}$ will be equal to 1.00. By contrast, in the case of a neuron whose RF is spatially invariant, the non-shifted alignment yields the highest correlation, resulting in $t_{\max}$ and $t_{\max}/D_{\text{eye}}$ of zero. A mean shift ratio was computed from the 36 different pairs formed by nine different RF maps.

Eye-position effects. A global influence of eye position can be observed on baseline activity in darkness or on visually evoked activity. To measure the effects on baseline activity, we counted the number of spikes present during the 400 ms fixation period preceding the onset of the first stimulus in each trial.

Analyses conducted on visually evoked activity were based on the number of spikes in the post-stimulus window for all locations evoking discharges above 50% of the maximum firing rate. For both sets of measurements, differences in firing rate between the nine fixation locations were screened with a distribution-free ANOVA. Neurons with a significant effect were further examined using a two-dimensional linear regression in order to determine if a simple oriented plane could accurately describe the observed variation. Analyses of visually evoked activity were restricted to cells whose RF remained within the boundaries of the mapped area at all eye positions, in order to avoid identifying artefactual eye-position effects. For this subpopulation, a comparison between results obtained with baseline and visually evoked activity showed similar proportions of cells with and without significant effects, and of cells with reliable planar gain field, in agreement with previous observations⁷. Baseline activity can therefore be considered a reliable indicator of eye-position gating, and the results reported for the full population were based on this parameter.

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False perception of motion in a patient who cannot compensate for eye movements

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We are usually unaware of the motion of an image across our retina that results from our own movement. For instance, during slow-tracking eye movements we do not mistake the shift of the image projected onto the retina for motion of the world around us, but instead perceive a stable world. Following early suggestions by von Helmholtz¹, it is commonly believed that this spatial stability is achieved by subtracting the retinal motion signal from an internal reference signal, such as a copy of the movement command (efference copy)²⁻⁴. Object motion is perceived only if the two differ. Although this concept is widely accepted, its anatomical underpinning remains unknown. Here we describe the case of a patient with bilateral extrastriate cortex lesions, suffering from false perception of motion due to an inability to take eye movements into account when faced with self-induced retinal image slip. This is indicated by the fact that during smooth-pursuit eye movements, he perceives motion of the stationary world at a velocity that corresponds to the velocity of his eye movement; that is, he perceives the raw retinal image slip uncorrected for his own eye movements. We suspect that this deficiency reflects damage of a distinct parieto-occipital region that disentangles self-induced and externally induced visual motion by comparing retinal signals with a reference signal encoding eye movements and possibly ego-motion in general.

R.W., a 35-year-old male, was admitted to our hospital in August 1996. He complained of vertigo and nausea that he had been experiencing for more than 6 years. Vertigo in this patient occurs either with the eyes tracking moving objects (for example watching his children on a carousel or playing computer games that require the observation of moving targets) or with the eyes fixed on a stationary object during translational ego-motion (such as watching objects through the lateral window of a moving car or train). It promptly disappears when the eyes are closed. On careful neurological examination, no abnormality was found except for a slight reduction in visual acuity of the left eye (see Methods for clinical details). Magnetic resonance imaging (MRI) revealed bilateral extrastriate cortex lesions (Fig. 1). The specific conditions under which vertigo in R.W. occurs led us to assume that it was selectively related to the processing of visual motion induced by ego-motion. In order to verify this hypothesis, we performed detailed psychophysical tests assessing R.W.'s perception of self-induced visual motion resulting from his smooth-pursuit eye movements.

Whenever smooth pursuit is carried out across a stationary background, the resulting slip of the background image on the retina has to be related to the eye movement in order not to be misinterpreted as background motion. According to the inferential theory of perception, visual stability during pursuit eye movements is the result of the comparison of a signal that reflects retinal image slip with an internal reference signal that encodes the eye movement¹⁻⁴. If this comparison were perfect, no pursuit-induced background movement would be perceived. However, although our visual system comes close to this ideal (see below), slight imperfections can be revealed in the form of the perception of a tiny and

usually non-disturbing movement of the stationary world. This illusionary background motion induced by pursuit eye movements is commonly referred to as the Filehne illusion⁵. The Filehne illusion can be measured by determining the amount of external background motion required to compensate for it, thus yielding the impression of a stationary background. At this point of subjective stationarity (PSS), the physical background motion is equal in magnitude and opposite to the direction of the Filehne illusion⁶. The smaller the illusion, the better our ability to cope with self-induced retinal image slip. Using a two-alternative forced choice paradigm with varying background velocities, we identified the PSS for horizontal pursuit eye movements as the one giving on average as many left as right responses⁷. In accordance with our previous work using similar stimulation conditions⁸, our measurements revealed that the illusionary motion perceived was indeed almost negligible in healthy subjects ($n = 18$) whose mean age matched that of R.W. This was indicated by the fact that background velocity at the PSS was close to 0 deg s⁻¹ over a broad range of eye velocities tested (Fig. 2). In R.W., however, stimulus velocity at the PSS was dramatically increased (Fig. 2) and moreover was dependent on the eye velocity preceding. Despite a considerable variability in his performance, there was a clear tendency towards higher Filehne

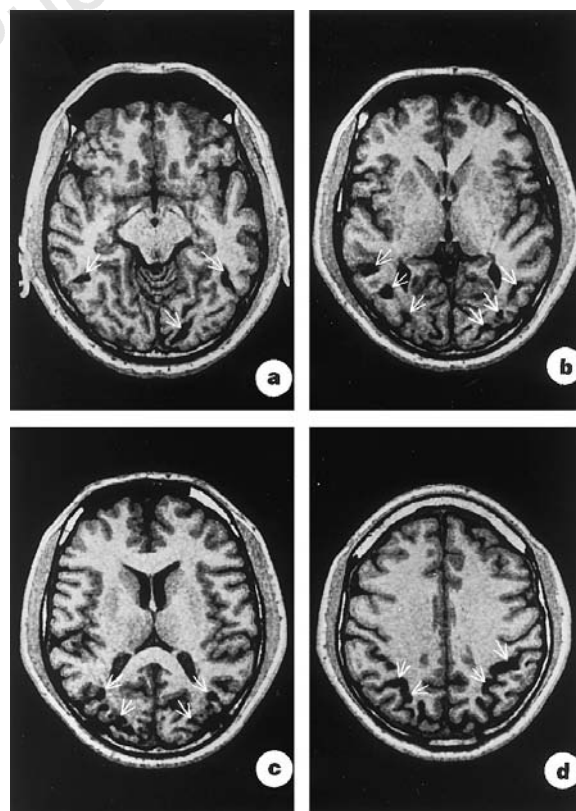


Figure 1 MRI shows bilateral cyst-like local widenings (arrows) of the sulci of the occipital lobe mainly affecting parts of areas 18, 19 and possibly 37 on the lateral aspect of the hemispheres and areas 18 and 19 on the inferior aspect (**a-c**). In addition, cortex in and around the intraparietal sulcus of the parietal lobes is involved (**d**). Axial 0.9-mm reconstructions parallel to the line connecting the anterior and posterior commissures (AC-PC line) are calculated from a high-resolution T1-weighted three-dimensional data set (3D-FLASH 30°, TR/TE = 30/5 ms, 1.5 tesla). T2-weighted images (not shown) were also consistent with sulcal widenings containing CSF. The lesions represent strictly cortical defects and local cortical atrophy without signs of progression. Subcortical white matter and basal ganglia are intact. Although the lesion pattern does not allow a definitive pathogenetic assignment, post-hypoxic cortical laminar necrosis seems to be most likely. As a possible cause, R.W. had suffered from a severe pertussis infection in early childhood which had required artificial respiration.

illusions with increasing eye velocities. Specifically, the linear regression plotted in Fig. 2 implies that R.W. did not perceive 'stationarity' unless background stimulus velocity roughly equalled target velocity—that is, unless the background image was stabilized on his retina.

In a first set of control experiments, we investigated whether R.W. suffered from a general deficit in retinal motion analysis also present in the absence of eye movements. We measured R.W.'s perception of visual motion that was not a consequence of self-motion but was instead due to motion in the environment. These tests, performed under stationary fixation, did not reveal any deficiency. Specifically, R.W. was as successful in detecting coherent motion in a random dot display⁹ as were normal controls (Fig. 3a). His ability to extract a two-dimensional shape from motion, as assessed by a motion-defined letter test¹⁰, was also unimpaired (motion threshold 0.093 deg s^{-1} as compared to a normal limit of 0.11 deg s^{-1} (refs 10, 11)). R.W. was also able to use retinal motion information to generate smooth eye movements, compensating for target motion, as indicated by pursuit eye movements of normal latency and gain (Fig. 3b). As a perceptual consequence of his intact smooth-pursuit eye movements, the acuity for moving Landolt targets (recently shown to be highly susceptible to even slight smooth-pursuit deficits¹²) was excellent in R.W. (binocular acuity was 1.0 both for a stationary Landolt target and a Landolt target

moving at speeds of up to 12 deg s^{-1}). In summary, these results suggested that R.W.'s ability to analyse object-induced retinal image motion for perceptual as well as for oculomotor purposes was unimpaired.

R.W. is, moreover, by no means generally impaired on motion analysis during ego-motion. If efferent information such as a signal encoding the eye movement is not required, his performance is normal. This conclusion is suggested by an experiment, in which we measured R.W.'s ability to detect a change in the velocity of the pursuit target. This task can be performed successfully by detecting the retinal image slip resulting from the change in target velocity. Indeed, R.W.'s sensitivity thresholds for both acceleration and deceleration of the pursuit target were indistinguishable from those of controls ($n = 6$, Fig. 4).

In summary, our observations on R.W. show that the ability to perceive a stationary world, despite self-induced visual motion, can be selectively impaired as a consequence of an extrastriate cortex lesion. R.W. interprets retinal image slip as object motion in extrapersonal space, irrespective of whether it results from motion in the environment or, alternatively, from his pursuit eye movements. Because retinal analysis of object motion is normal in R.W., it must be the necessary comparison with an internal signal encoding the eye movement, which is impaired. Either the appropriate reference signal is missing or the comparison itself may no longer be

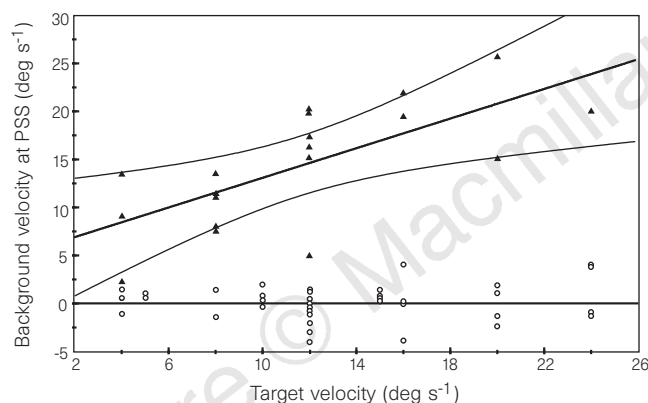


Figure 2 Perception of pursuit-induced visual motion in patient R.W. (filled triangles) and 18 control subjects (50 individual measurements represented by open circles, partially lying on top of each other). The background stimulus velocity that is perceived as stationary (PSS, point of subjective stationarity), that is, the estimate of the Filehne illusion, is plotted for the different pursuit target velocities varied from 4 to 24 deg s^{-1} . Target motion was always to the right. Positive values of background velocity at PSS indicate background movement in the same direction as the eyes. The higher the background velocity at PSS, the less the resulting retinal image slip velocity allowing the percept of stationarity and, thus, the less the ability to cope with self-induced visual motion. In the control subjects stimulus velocity at PSS is always close to 0 deg s^{-1} . Hence perceived background motion is tightly related to visual motion in extrapersonal space, that is, stationarity is preserved despite increasing retinal image shifts with growing target velocities. In R.W., however, perceived motion reflects image movement on the retina. The linear regression (with 99% confidence bands) suggests that R.W. is deprived of a sense of stationarity unless the background image is stabilized to some degree on his retina. Accordingly, he will experience Filehne illusions in the order of the eye velocity prevailing.

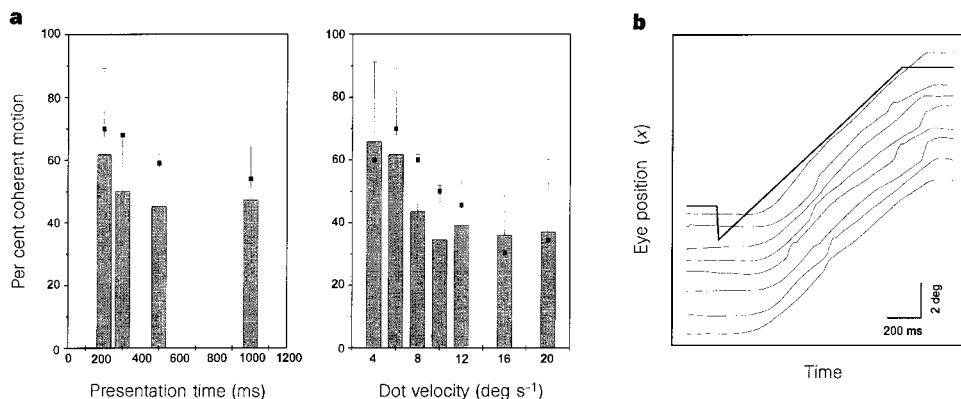


Figure 3 Evidence for unimpaired analysis of retinal motion in patient R.W. **a**, Detection of coherent visual motion in a random dot display. Threshold values of per cent coherence as function of stimulus presentation time (left panel, dot velocity, 6 deg s^{-1}) and dot velocity (right panel, presentation time, 200 ms), respectively. The results obtained from R.W. (represented by single bars) as compared to a group of 12 healthy controls (means/standard deviations) reveal no deficit and further show the same dependency on the two parameters varied.

b, Records of individual trials of smooth-pursuit eye movements elicited using a step-ramp paradigm (step amplitude, 2 deg; ramp velocity, 10 deg s^{-1}). Eye movements were recorded with a high-resolution infrared reflection system at a sampling frequency of 200 Hz. Horizontal ramps to the right (upper four trials) and left (lower four trials) were unpredictably intermingled. Only the x-components of eye position are depicted.

possible. In either case, the location of the bilateral lesions revealed by MRI suggests that neural networks contributing to this comparison are located in the parieto-occipital lobe. This conclusion is in line with our recent demonstration of a parieto-occipital event-related potential that reflects the percept of self-induced visual motion⁸. We propose that the region destroyed in patient R.W. may involve cortex homologous to monkey area MST^{13,14}, and moreover that the functional deficit may be a direct consequence of this loss. The reason is that single-unit studies on monkeys have suggested that the ability to ignore self-induced retinal image slip might be confined to this late stage of cortical processing of visual motion while being absent in earlier stages including area V5^{15,16}. The precise localization of human area MST is still a matter of debate. However, there is little doubt that it must lie immediately adjacent to human motion processing area V5, whose location close to the ascending limb of the inferior temporal sulcus is well established^{17,18}. This region is part of the lesions in R.W., which therefore most probably not only involve putative human area MST but also area V5. In view of the probable involvement of V5, our finding of a normal analysis of retinal image motion, a function generally attributed to area V5, is puzzling. A possible explanation of this seeming paradox is offered by the fact that R.W. acquired the lesions probably in his early childhood, possibly early enough to allow spared elements of cortical motion processing to step in. Indeed, R.W. would not suffer from vertigo if the cortical processing of retinal image slip had not been maintained. □

Methods

Clinical details. Although R.W. described vertigo and nausea only for the past 6 years, his history revealed several peculiarities that suggest that he had been suffering from a loss of visual stability for a much longer time, possibly since his early childhood. For instance, he had never tried to acquire a driver's licence because he had been insecure in judging speed or distance of objects when

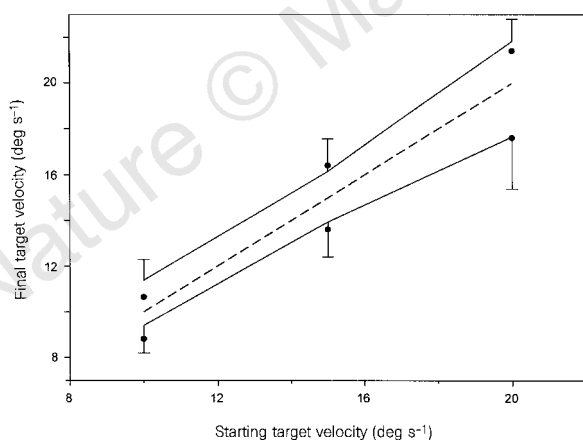


Figure 4 Performance of R.W. (dots) and 6 control subjects (means and standard deviations) on a task assessing the sensitivity to changes in the velocity of a moving target. The observers were instructed to track a target which started to move to the right at a fixed velocity, changed its velocity after a variable time interval and continued moving at the final speed for the rest of the target sweep. Using a two-alternative forced choice paradigm, thresholds were determined both for acceleration and deceleration by means of two separate staircase procedures running simultaneously within each session. The upper solid curve plots the final target velocity acquired after just perceptible accelerations, the lower solid curve the final target velocity after just perceptible decelerations of the target. The curves connect the mean thresholds for acceleration and deceleration, respectively, of the 6 controls. For the sake of clarity, standard deviations are plotted only for non-overlapping directions. The minimum perceptible acceleration and deceleration for a given starting target velocity can be deduced from this graph by measuring the deviation of the final target velocity from the starting target velocity (dashed line in between the two solid curves).

travelling at high velocities, and during childhood, a striking insufficiency in ball games had always been in exceptional contrast to above-average results in athletics. Of course, both the analysis of external objects during car driving as well as the pursuit of balls in ball games involve goal-directed eye movements and consequently the need to handle self-induced retinal image slip.

On examination, the common causes of vertigo, such as peripheral or central vestibular dysfunction, were carefully excluded. For instance, there was no hearing loss, tinnitus or any kind of nystagmus. Caloric testing did not reveal any asymmetry in response and the vestibulo-ocular reflex was normal in terms of gain and phase. Pursuit eye movements, saccades and optokinetic nystagmus were intact, both clinically and on electronystagmographic recording. Electroencephalography demonstrated no focal slowing or paroxysmal discharge and thus provided no electrophysiological correlate of vestibular epilepsy. Moreover, R.W.'s failure to account for self-induced retinal motion was highly selective because other visual functions did not reveal any relevant disturbance. Specifically, no visual field defect was found in perimetric tests and colour vision, as assessed by Ishihara plates, was unimpaired. The only irregularity found was a slight reduction in acuity for the left eye (0.8 as compared to 1.0 for the right eye, resulting in a binocular acuity of 1.0). It had been attributed to a mild convergent strabism, which was surgically corrected in August 1995.

Psychophysical tests. With the exception of the motion-defined letter test (refs 10, 11; see ref. 11 for the specific parameters chosen), all other psychophysical tests followed an adaptive staircase procedure (PEST)¹⁹, forcing the observer to select one of two alternative responses in the individual trial. Thresholds, as defined below, were determined by means of a probit analysis²⁰ with subsequent chi-square goodness-of-fit tests performed on the responses obtained from at least 40 trials in each session. Stimuli were presented on a 19-in computer monitor (Mitsubishi, frame rate 72 Hz, 1,280 × 1,024 pixel) in a dark (Filehne illusion, detection of velocity steps) or a brightly lit (static and dynamic visual acuity, coherent motion stimuli) experimental room. Viewing distance was 225 cm for the acuity measurements and 57 cm in the other experiments. During all tests, eye movements were monitored using an infrared iris reflection system (Amtech). Recordings were stored and analysed on-line at a sampling rate of 72 Hz by a workstation which also controlled the presentation of the stimuli. Deviations of eye position from the position of the given target exceeding 2 deg were fed back acoustically as errors and the corresponding trials were discarded. Off-line analysis of the recordings revealed no difference in oculomotor performance obtained from R.W. as compared to the control groups. Specifically, eye velocity was always within the range of group means $\pm$ one standard deviation, and hence was verified not to account for any differences in the behavioural results.

Filehne illusion. Measurements of the Filehne illusions were performed as recently described by us⁷. Briefly, pursuit was elicited by a red dot (diameter 10 min of arc) which moved to the right at a constant velocity spanning a visual angle of 30 deg. Temporally located in the middle of the target sweep a background pattern was presented for 300 ms. This background stimulus subtended 27×27 deg of visual angle and consisted of 350 white dots (diameter 15 min of arc, local contrast 0.01). Subjects were asked to report the direction of perceived background motion which was varied by the psychophysical procedure. The PSS was defined as the background velocity that resulted in 50% left and 50% right responses after repeated presentation. Measurements started with the background stimulus velocity being 4 deg s^{-1} and were randomly interleaved with constant stimuli ($\pm 4 \text{ deg s}^{-1}$, respectively).

Coherent motion stimuli. The ability to detect coherent motion in a random dot field was tested by determining the minimum percentage of coherently moving dots required to discriminate this field from a second random dot field containing only non-coherently moving dots. The 2 random dot fields were borderless squares of 12×12 deg each and were presented simultaneously with their inner borders 2 deg left and right, respectively, of the fixation spot. Each random dot field contained 400 dots (diameter 7 min of arc, local contrast 0.1) moving at a specified velocity for a limited lifetime of 100 ms. For each trial a common direction of movement was chosen randomly between 0 and 360° for the coherently moving dots. The field containing coherent motion was chosen at random and the percentage of coherently moving dots was varied according to the adaptive staircase procedure. A coherence threshold was determined at which subjects responded correctly 75% of the time (where 50% correct is the

performance expected by chance). Presentation time and dot velocity were varied systematically as described in the results section. Note that several features of the design chosen make it more demanding than similar tests published⁹, leading to comparatively high thresholds: these features involve the large number of dots, short dot lifetime, and particularly the need to compare two spatially separated random dot fields instead of detecting motion coherence in only one field.

Static and dynamic visual acuity. Resolution thresholds were measured both for stationary Landolt Cs (static visual acuity) and for moving Landolt Cs to be tracked by eye movements (dynamic visual acuity). Again, subjects had to press one of two buttons according to two possible positions of the Landolt C gap (either left or right) in this set of experiments. Optotypes were presented on a local background (white circle, luminance 60 cd m⁻², diameter equalling diameter of the Landolt C plus 2 × 20 min of arc) on an otherwise grey screen (luminance 7.8 cd m⁻²). In the measurements of static acuity, trials started with a 500-ms presentation of the stationary local background followed by the Landolt C being visible for 250 ms. Dynamic acuity was determined using a ramp paradigm, with the local background moving in a rightward horizontal direction at a given velocity (visual angle 8 deg). Unpredictably within this sweep, the Landolt C was added to the local background and participated in the movement for 250 ms. Acuity thresholds were defined as the Landolt C gap resulting in 75% correct responses, spatial resolution of the monitor was enhanced by means of anti-aliasing techniques.

Detection of velocity steps. In this task the observers were instructed to track a target (diamond, length 10 min of arc, local contrast 0.1 as compared to the otherwise dark screen) which started to move to the right at a fixed velocity, changed its velocity after a variable interval (at least 800 ms) and continued moving at the final speed for another 500–1,400 ms (total ramp length, 30 deg). Subjects were asked to indicate whether the target had accelerated or decelerated during the sweep. Thresholds (defined by 75% right and left responses, respectively) were determined both for acceleration and deceleration by means of two separate staircase procedures running simultaneously within each session.

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Ligand-induced changes in integrin expression regulate neuronal adhesion and neurite outgrowth

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Receptors of the integrin family are expressed by every cell type and are the primary means by which cells interact with the extracellular matrix. The control of integrin expression affects a wide range of developmental and cellular processes, including the regulation of gene expression, cell adhesion, cell morphogenesis and cell migration^{1–3}. Here we show that the concentration of substratum-bound ligand (laminin) post-translationally regulates the amount of receptor ($\alpha_6\beta_1$ integrin) expressed on the surface of sensory neurons. When ligand availability is low, surface amounts of receptor increase, whereas integrin RNA and total integrin protein decrease. Ligand concentration determines surface levels of integrin by altering the rate at which receptor is removed from the cell surface. Furthermore, increased expression of integrin at the cell surface is associated with increased neuronal cell adhesion and neurite outgrowth. These results indicate that integrin regulation maintains neuronal growth-cone motility over a broad range of ligand concentrations, allowing axons to invade different tissues during development and regeneration.

Cell migration is critical to the development of all embryonic tissues. For cell migration to occur, a precise balance must be maintained between adhesion to the substratum, which is necessary to generate mechanical force, and de-adhesion, which is required to change position. For many cell types^{4–7}, substratum-bound extracellular matrix (ECM) molecules support optimal motility over a very limited range of ECM surface densities. As the concentration of substratum-bound molecules or the number of cellular receptors increases, the strength of cell attachment to the substratum increases until cells are attached too strongly to migrate⁸. In contrast to other migratory cell types, neurons both *in vivo*⁹ and *in vitro*¹⁰ (Fig. 1a) extend neurites over a wide range of ECM ligand concentrations, suggesting that they regulate their degree of adhesion to the ECM. The capacity of sensory and motor neurons to regulate cell–matrix interactions requires that they have a mechanism for modulating either the expression or function of ECM receptors that is not seen in other non-invasive cell types. Neurons could alter their interactions with the matrix to preserve growth-cone motility either by changing receptor affinity¹¹ or by regulating the amount or the subcellular distribution of receptors. These possibilities can be distinguished by examining the number of ECM receptors that are expressed under conditions of high and low availability of ligand.

The hypothesis that neurons respond to low availability of a growth-promoting ECM ligand such as laminin by increasing their expression of receptor, thereby strengthening the interaction with the substratum, predicts that the levels of laminin receptor expressed by a neuron should increase when the amounts of substratum-bound laminin decrease. Dorsal root ganglion neurons were cultured overnight on substrata containing a tenfold difference in bound laminin or on fibronectin, as a control for the absence of laminin (Fig. 1b), and analysed for expression of integrin $\alpha_6\beta_1$ (the only laminin-specific integrin known to be expressed by sensory

neurons¹²⁻¹⁴). In chick, unlike dorsal root neurons from other species¹³, α_6 is expressed exclusively with the β_1 subunit to form a laminin receptor; no bands of appropriate molecular size for the β_4 chain were co-precipitated with α_6 antibodies from either metabolically labelled or surface-biotinylated neurons. Laminin is the only reported ligand for $\alpha_6\beta_1$. Antisera against α_6 integrin block adhesion of chick dorsal root neurons to laminin ($77 \pm 8\%$ of control adhesion in the presence of non-immune sera, $n = 3$ with triplicate determinations each experiment) but do not affect neuronal adhesion to fibronectin ($102 \pm 5\%$ control adhesion). This level of inhibition is similar to that seen using the same antibody in chick ciliary neurons, where α_6 is also primarily associated with β_1 and the principal laminin receptors are integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$ (ref. 15).

Surprisingly, when neurons were cultured overnight on different concentrations of laminin, the amounts of α_6 integrin RNA and total protein were not increased under conditions of low laminin availability (Fig. 2a, b). Rather, in cells cultured on low levels of laminin, the amount of α_6 integrin RNA and total α_6 integrin protein expressed were consistently lower than for the other conditions. These results suggest that if receptor regulation allows neurons to extend neurites on a wide range of ligand densities, the regulation is likely to occur post-translationally.

To explore the possibility of post-translational integrin regulation in neurons, the cell-surface receptor levels were examined. In marked contrast to the observed decrease in integrin RNA and total protein accumulation, the amount of α_6 integrin expressed at

the cell surface was greatly increased when neurons were cultured on substrata containing low levels of laminin. Neurons experiencing low ligand availability expressed four to five times more α_6 integrin protein at the cell surface than cells cultured either on high levels of laminin or on fibronectin (Fig. 2c, Table 1). Similar results were obtained for the expression of RNA, total protein and surface protein of $\alpha_3\beta_1$ integrin, which functions as a receptor for both laminin and fibronectin (data not shown). The increase in cell-surface α_6 protein at the same time that levels of α_6 RNA and total α_6 protein decrease represents a new form of post-translational integrin regulation. Integrins undergo a variety of post-translational modifications, including glycosylation, proteolytic processing, and changes in affinity for ligand binding¹¹. The regulation of integrin reported here is both compensatory (integrin α_6 expressed at the cell surface is high when availability of the ligand is low, and low when the ligand is abundant), and is not seen in cells cultured on

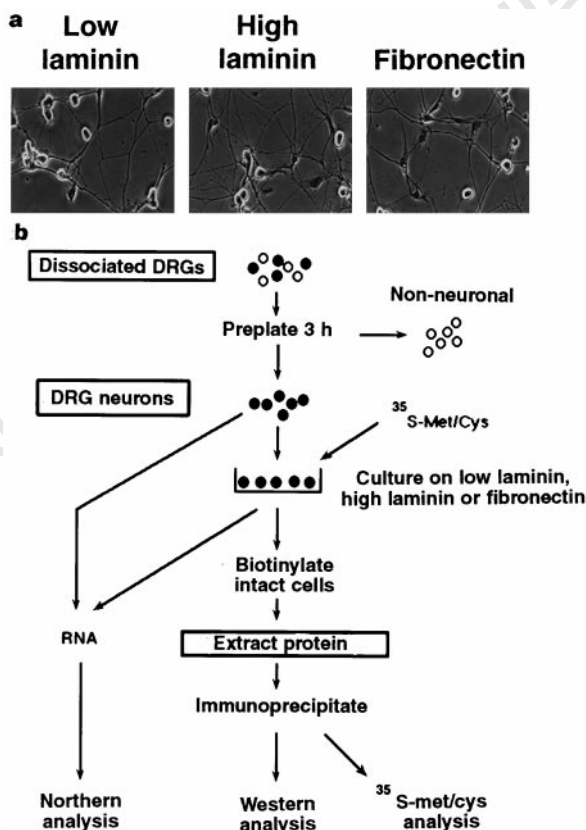


Figure 1 Characterization of dorsal root ganglion (DRG) neurite outgrowth and integrin expression when cultured on different substratum densities of laminin or on fibronectin. **a**, Neurite outgrowth from dissociated embryonic DRG neurons cultured for 20 h on either low (30 ng cm^{-2}) or high (300 ng cm^{-2}) substratum densities of laminin is approximately equivalent to neurite outgrowth on fibronectin. **b**, Schematic representation of the quantitative analysis of integrin RNA, total receptor protein and cell-surface protein from DRG neurons cultured on different substrata.

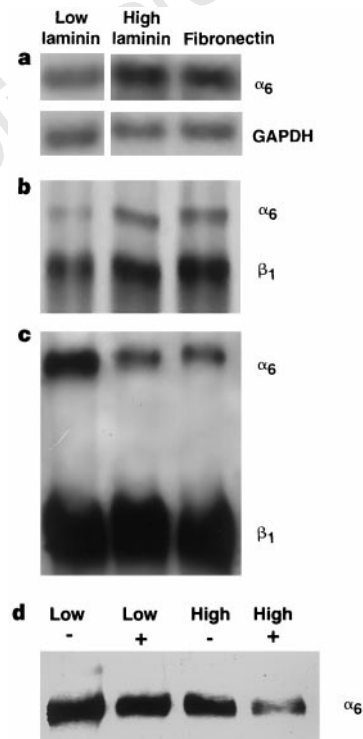


Figure 2 Analysis of α_6 integrin RNA, total α_6 integrin protein and surface α_6 integrin protein from cells cultured on different concentrations of ligand. **a**, Northern analysis indicates that α_6 integrin RNA decreases significantly in neurons cultured on low laminin relative to neurons cultured on either high laminin or fibronectin. Blots were stripped and reprobed for GAPDH as a loading control (densitometry of exposed films (Table 1)). **b**, Neuronal proteins were metabolically labelled during culture (Fig. 1b), and immunoprecipitated with an antibody specific for α_6 integrin under conditions that co-precipitate the associated β_1 subunit. Less α_6 integrin protein was present in neurons cultured on low laminin relative to neurons cultured on either high laminin or fibronectin (Table 1). **c**, Four- to fivefold more α_6 integrin protein is expressed at the cell surface in cells cultured on low laminin than in neurons cultured on either high laminin or fibronectin. Surface proteins were labelled with biotin, immunoprecipitated and analysed on western blots. **d**, Lifespan of α_6 integrin protein at the cell surface decreases when cells are cultured on high laminin. Neurons that had been cultured overnight on low laminin to increase α_6 integrin expression and control for any effects of cell culture were briefly replated on substrata containing either low or high laminin in the presence (+) or absence (-) of 1 mM primaquine to retard secretion of receptor to the cell surface. The α_6 integrin protein remaining at the cell surface after 1 h was labelled with biotin, immunoprecipitated and analysed on western blots. Relative amounts of α_6 integrin protein measured in this example: Low (-), 1.0; Low (+), 0.87; High (-), 0.64; High (+), 0.16.

fibronectin in the absence of the ligand for α_6 integrin. This finding indicates that changes in the cell-surface concentrations of laminin receptor are dependent on the presence of laminin.

What mechanism could lead to an increase in protein at the surface under conditions where the levels of α_6 RNA and total integrin α_6 protein decrease? The possibility that subpopulations of dorsal root ganglion neurons with intrinsically different levels of integrin expression were being selected on different substrata was tested by culturing neurons overnight on substrata containing low levels of laminin, removing the neurons from the dish, and replating on both high- and low-laminin substrata. Within 1 h there was a clear difference in the surface integrin levels between cells replated on high and low levels of laminin (Fig. 2d). When primaquine was added to block secretion of new integrin¹⁶, and thereby measure the lifespan of the protein at the cell surface in isolation, the rate at which integrin was removed from the cell surface was much greater in cells replated on high laminin than for those replated on low laminin (cell-surface receptor levels declined approximately 6.5-fold faster in cells replated on high levels of laminin). This rapid change in cell-surface α_6 integrin expression in cells that had received identical overnight treatment argues against the possibility that subpopulations of dorsal root ganglion neurons with different levels of integrin expression are being selected by our culture conditions. Rather, differences in the lifespan of the receptor at

the cell surface in response to different amounts of laminin indicate that the availability of ligand determines the levels of receptor expressed at the cell surface by altering the rate of receptor internalization or degradation^{17,18}. Internalization of α_6 -containing receptors may be mediated by sequences in the cytoplasmic domain of the α_6 subunit¹⁹. For other receptor types, the effect of receptor regulation by ligand concentration is to maintain a cell's responsiveness over a broad range of ligand concentrations²⁰, suggesting that the regulation of integrins by ECM ligands maintains growth-cone motility throughout changing extracellular environments.

The effect of altered integrin expression on neuronal cell behaviour was determined by examining both the adhesion and neurite outgrowth of cells cultured overnight on different substrata to alter their integrin expression (Fig. 3). Cells expressing high levels of receptor at the cell surface (cells cultured overnight on low laminin) exhibited much greater initial adhesion to both laminin and fibronectin than cells expressing lower levels of receptor at the cell surface (cells cultured overnight on either high laminin or fibronectin before the adhesion assay; Fig. 3a). The rate of neurite outgrowth under conditions of low ligand availability was also greatly increased in cells with increased surface expression of laminin receptors. A three- to fourfold increase in the initial number of neurons extending neurites on low-laminin substrata

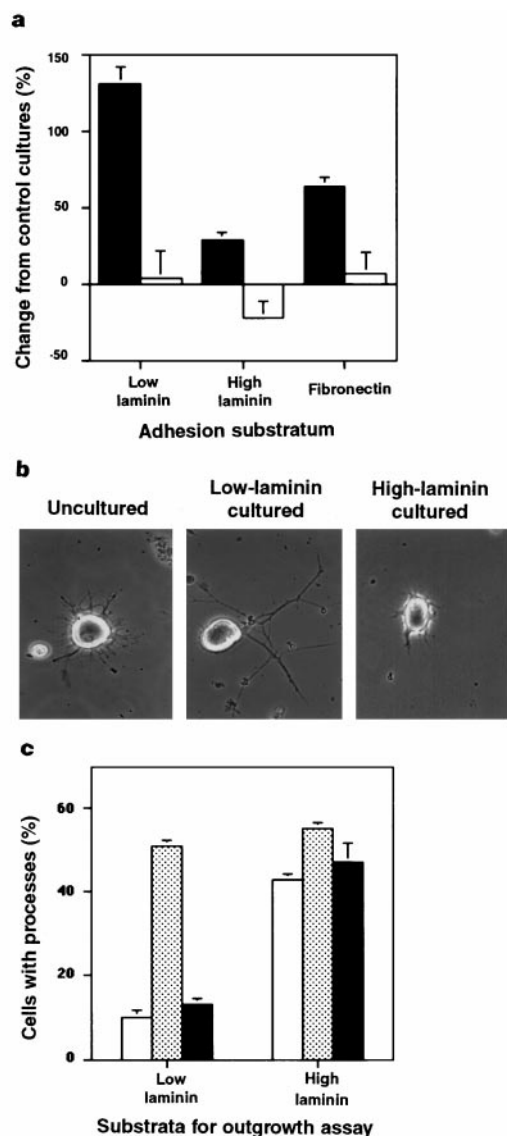


Figure 3 Increased surface expression of integrin receptors increases both cell adhesion and rate of neurite outgrowth. **a**, The number of cells adhered to either low laminin, high laminin or fibronectin was determined for cells that had been cultured overnight on either low laminin, high laminin or fibronectin as a control substratum. The bars indicate the percentage difference between adhesion of low-laminin (black bars) or high-laminin (white bars) cultured cells and adhesion of cells from control cultures. Differences in adhesion between the high- and low-laminin cultured cells for all three adhesion substrata were significant at $P < 0.025$ or better (t -test). Numbers of cells retained on low laminin, high laminin- and fibronectin-containing substrata were $1,455 \pm 404$, $5,584 \pm 498$ and $7,025 \pm 874$, respectively, for cells previously cultured on low laminin, and 708 ± 272 , $3,469 \pm 508$, and $4,716 \pm 568$, respectively, for high-laminin cultures. Increased adhesion to fibronectin in the low-laminin cultured cells can potentially be explained by increased surface expression of other integrin receptors (such as $\alpha_3\beta_1$ and $\alpha_7\beta_1$) that function as both laminin and fibronectin receptors. **b**, Cells expressing high levels of surface integrin (low-laminin cultured) extend neurites within 3 h of incubation on low-laminin substrata, whereas cells expressing lower levels of integrin at the surface (uncultured and high-laminin cultured) do not. **c**, The percentage of cells that extended neurites greater than one cell diameter during a 3-h incubation on low laminin was much higher for cells that had been previously cultured overnight on low laminin to increase the surface expression of laminin receptors. Of cells cultured on low laminin overnight (expressing high levels of α_6 integrin protein at the cell surface), $51 \pm 1.5\%$ ($n = 906$) extended axons during a 3-h incubation on low laminin, compared with $13 \pm 1.5\%$ ($n = 1,508$) for cells cultured overnight on high laminin, and $10 \pm 1.7\%$ ($n = 1,130$) for uncultured cells. The percentage of cells extending axons greater than one cell diameter after 3 h on high laminin was similar for all three conditions: $43 \pm 1.2\%$ ($n = 1,040$) for uncultured (white bars), $55 \pm 1.5\%$ ($n = 891$) for low-laminin (stippled bars), and $47 \pm 4.6\%$ ($n = 679$) for high-laminin cultured (black bars).

was observed for cells expressing high levels of laminin receptor relative to control conditions (Fig. 3b, c). These results suggest that the regulation of surface receptors can compensate for low ligand availability to increase neuronal attachment to the substratum and maintain growth-cone motility.

The surface expression of integrins in non-neuronal cells can be altered by several factors that also affect cellular differentiation, cell morphology, the expression of EM components, and the expression of ECM-modifying enzymes^{21,22}. In contrast to situations in which integrin regulation may be part of a larger change in cell state, the regulation of integrins we observed occurs in terminally differentiated neurons under conditions that do not appreciably affect cell morphology (Fig. 1a), providing strong evidence for the direct regulation of neuronal integrins by the ECM (Fig. 4). As neuronal growth cones migrate during development, they encounter a dynamic extracellular environment in which both the levels and types of extracellular molecules change rapidly. Sensory and motor growth cones invade a wide range of tissues over a relatively long period of developmental time, and therefore need to preserve the capacity to migrate in the face of significant alterations in the extracellular landscape. Recent data suggest that newly synthesized proteins are inserted preferentially at the growth cone of neurons and at the leading margin of other migrating cell types^{23–25}. With a constant supply of new receptor at the growth cone, regulation of cell-surface receptor levels by ligand-dependent endocytosis would allow growth cones to respond rapidly to different environmental conditions, without requiring alterations in the overall synthesis of receptor. The regulation of integrins by the ECM may be exclusive to neurons and other highly invasive cell types that maintain the capacity of a cell to migrate when challenged with changing amounts and composition of ECM in the different tissues they encounter. □

Methods

Cell culture and substratum preparation. Dorsal root ganglion cells (DRGs) from chicks at embryonic day 12 were preplated to remove non-neuronal cells and cultured overnight on glass coverslips²⁶. Before application of neurons,

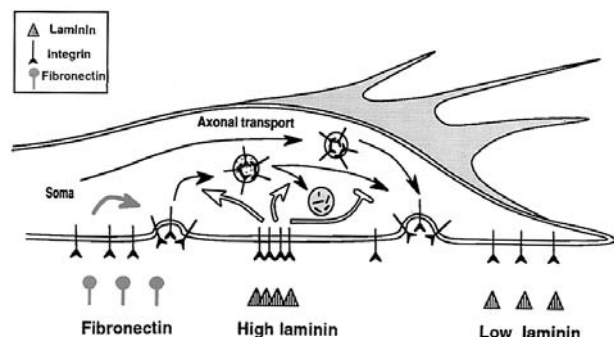
Table 1 Relative amounts of α_6 integrin receptor message, total protein and surface protein

	Low laminin/high laminin*	Fibronectin/high laminin†
Total α_6 RNA	0.62 (0.66–0.58)‡	1.15 (1.24–1.07)
Total α_6 protein	0.47 (0.62–0.35)‡	0.91 (1.1–0.75)
Surface α_6 protein	4.5 (6.4–3.2)	1.08 (1.14–1.03)

Cells were cultured on low laminin, high laminin and fibronectin. The means and 95% confidence intervals were calculated from the mean and s.e.m. of the log (low laminin/high laminin) or log (fibronectin/high laminin) ratios from at least three independent experiments. * Ratios of RNA, total protein and surface protein are statistically different from 1 at $P < 0.01$, < 0.1 and < 0.01 , respectively (t -test).

† None of the ratios are statistically different from 1.

‡ The difference in total receptor protein observed between high and low laminin was more pronounced than would be predicted by the difference in RNA alone ($P < 0.1$ that the low laminin/high laminin ratio for RNA equals the ratio for total protein; t -test).



coverslips were coated for 1 h with laminin at $1 \mu\text{g ml}^{-1}$ or $20 \mu\text{g ml}^{-1}$ or fibronectin at $20 \mu\text{g ml}^{-1}$ in PBS, and rinsed extensively. This resulted in concentrations of bound laminin at either 30 (low laminin) or 300 (high laminin) ng cm^{-2} . Binding of laminin to coverslips and 96-well plates (below) was determined by inclusion of ^3H -labelled laminin at known specific activity, extraction with 10% SDS, and scintillation counting. Approximately equal numbers of cells ($\pm 15\%$) were recovered from all three substrata.

RNA analysis. RNA was isolated from approximately 10^7 neurons by lysing the cells *in situ* in a phenol/SDS buffer (TriReagent; Molecular Research Center). RNA concentration was determined by ultraviolet absorption at 260 nm; $10 \mu\text{g}$ samples were analysed on agarose gels and transferred to nylon membrane by pressure blot (Stratagene) using standard procedures. Integrin α_6 and GAPDH transcripts were detected with ^{32}P -labelled cDNA probes derived from the cloned chicken α_6 subunit that recognize a single band of appropriate molecular size on northern blots²⁷. Films and blots were quantified using BioRad GS-700 imaging densitometer, GS-363 phosphorimager and NIH image 1.55 analysis software.

Protein analysis. Total protein was metabolically labelled by preincubating the cells for 3 h in cysteine/methionine-free medium to deplete internal stores and increase labelling efficiency, then labelling during an overnight culture in the presence of $50 \mu\text{Ci ml}^{-1}$ of ^{35}S methionine and ^{35}S cysteine (Amersham) in cysteine/methionine-free medium. Cell-surface receptor was labelled with biotin and immunoprecipitated using antibodies specific for α_6 integrin²⁷ and α_3 integrin (data not shown). Immunoprecipitated proteins were size fractionated under reducing conditions on acrylamide gels and electrophoretically transferred to nitrocellulose membrane using standard protocols. ^{35}S -labelled proteins were detected by autoradiography. Biotin-labelled proteins were detected using strep-Avidin conjugated to HRP and a chemoluminescent reagent (Pierce) followed by exposure to film. Ratios of protein determined from quantization of film were similar to those obtained by scintillation counting of ^{35}S -labelled bands excised from blots and to those obtained by direct phosphorimaging of ^{35}S or chemoluminescently detected proteins. To determine the lifespan of integrin at the cell surface (Fig. 2d), neurons were cultured overnight on low-laminin substrata to increase the expression of integrin at the cell surface and decrease the percentage of total integrin protein in intracellular pools. Cells were removed from the plates by gentle scraping after brief treatment with calcium-free media at $0-4^\circ\text{C}$ and replated at 37°C on substrata containing either high or low laminin in the presence or absence of 1 mM primaquine to retard secretion of protein to the cell surface. After 1 h, culture plates and any cells that had detached from the plates were placed at $0-4^\circ\text{C}$, and integrin protein remaining at the cell surface was labelled with biotin, immunoprecipitated and analysed as above.

Cell adhesion and outgrowth assays. To manipulate surface integrin levels, cells were cultured on either high laminin, low laminin or fibronectin overnight and removed from the plates as above. To determine short-term adhesion, 96-well plates (Dynatech) were coated with laminin or fibronectin for 2 h at concentrations of applied protein sufficient to result in surface densities of bound protein similar to those used for culturing. The plates were rinsed, blocked with PBS containing 5 mg ml^{-1} BSA and 0.2% sodium azide for 1 h and rinsed extensively. Cells were applied at 10,000 cells per well and allowed to adhere for 3 h at 37°C . To determine the contribution of integrin $\alpha_6\beta_1$ to neuronal adhesion to laminin, antisera against the extracellular domain of α_6

Figure 4 Summary of the effect of different ligand densities on the surface expression of integrins in growing neurons based on our results and previous work^{6,16–18,28,29}. When ligand density is low (low laminin), receptor is expressed at the cell surface in relatively high amounts, reflecting the constitutive levels of receptor synthesis, secretion and reuptake. When ligand density is high (high laminin, open arrows), receptor is removed from the surface through either targeted endocytosis and sequestration or degradation. In the absence of ligand (fibronectin, grey arrows), unbound receptor is also removed from the surface^{6,16–18}.

(ref. 15) or normal rabbit serum were included in the adhesion assay at 10%. After 3 h, wells were rinsed once with 100 μ l PBS and then incubated for 10 min in PBS containing 2 μ g ml⁻¹ 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes) followed by detection of fluorescein emission using a microplate fluorescence reader (Cytofluor II; Bioscience/Millipore). Adhesion of high- and low-laminin cultured cells from four independent experiments is presented as a percentage change from the adhesion of control cultured cells, that is [(cells adhered from low- or high-laminin cultures) - (cells adhered from fibronectin cultures)] / (average cells adhered from fibronectin cultures). To examine the effect of increased integrin expression on neuronal outgrowth, cells were cultured overnight on either high or low laminin to alter integrin expression, collected as above, incubated for 3 h on low-laminin substrata and fixed. Control (uncultured) cells were isolated from embryos, incubated for 3 h on low-laminin substrata and fixed. Cells with processes greater than one cell diameter were counted in three independent experiments.

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Anterograde transport of brain-derived neurotrophic factor and its role in the brain

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The role of neurotrophins as target-derived proteins that promote neuron survival following their retrograde transport from the terminals to the cell bodies of neurons has been firmly established in the developing peripheral nervous system. However, neurotrophins appear to have more diverse functions, particularly in the adult central nervous system. Brain-derived neurotrophic factor (BDNF), for example, produces a variety of neuromodulatory effects in the brain that are more consistent with local actions than with long-distance retrograde signalling. Here we show that BDNF is widely distributed in nerve terminals, even in brain areas such as the striatum that lack BDNF messenger RNA, and that inhibition of axonal transport or deafferentation depletes BDNF. The number of striatal neurons that contain the calcium-binding protein parvalbumin was decreased in BDNF^{+/−} and BDNF^{−/−} mice in direct proportion to the loss of BDNF protein, which is consistent with anterogradely supplied BDNF having a functional role in development or maintenance. Thus the anterograde transport of BDNF from neuron cell bodies to their terminals may be important for the trafficking of BDNF in the brain.

Nerve growth factor (NGF) and related members of the neurotrophin family promote the survival and phenotypic maturation of many types of neurons^{1,2}. In the developing peripheral nervous system, the actions of these factors are thought to be mediated by their retrograde transport from target tissues to the cell body, and null mutations of NGF, BDNF, neurotrophin (NT)-3 and NT-4 produce selective losses of autonomic and sensory neurons^{3–6}. However, the absence of gross neuronal losses in the brains of such null mutant mice, and the persistence of high levels of neurotrophin expression in the mature nervous system^{7,8}, suggest that neurotrophins have other functions in the central nervous system (CNS). For example, BDNF can rapidly modulate neurotransmitter synthesis, metabolism and release, postsynaptic ion channel fluxes, neuronal firing rates, and the long-term synaptic potentiation of neurons in the adult CNS^{9–12}. The temporal and spatial characteristics of these effects are more consistent with local actions of BDNF than with signalling mediated by long-distance retrograde transport.

Our observations and recent immunocytochemical evidence^{13,14} show that the endogenous BDNF protein in brain is not confined to neuronal cell bodies, being also distributed widely in the neuropil, including nerve terminals and preterminal axons. The absence of BDNF mRNA in some areas where the neuropil stains strongly for BDNF protein further suggests that it may be anterogradely transported by axons to the terminals of BDNF-expressing neurons. For example, we have detected substantial levels of BDNF protein in the adult rodent striatum¹⁵, despite the virtual absence there of BDNF mRNA^{7,8}. It is unlikely that retrograde axonal transport from striatal targets is the source of BDNF, as cells in these areas (globus pallidus, entopeduncular nucleus, and pars reticulata of the substantia nigra) do not express BDNF mRNA. In contrast, the cortical, nigral,

amygdala and thalamic neuron groups that project to the striatum contain high levels of BDNF mRNA^{2,7,8,16}.

Neither BDNF mRNA nor BDNF protein were detected in neuronal cell bodies within the rostral neostriatum of normal rats (Fig. 1). Instead, BDNF immunoreactivity was present in small, punctate profiles throughout the striatal neuropil. In the cortex, intense expression of BDNF mRNA was found in the neuronal perikarya of layers 2, 3, 5 and 6 (Fig. 1). BDNF immunoreactivity was also evident in neuronal cell bodies in these layers, as well as in the neuropil. In the substantia nigra, BDNF mRNA was detected in the pars compacta but not in the underlying pars reticulata (Fig. 1).

After colchicine treatment to inhibit axonal transport, BDNF immunoreactivity was markedly elevated in the cell bodies of all principal striatal afferents, including the neocortex, the pars compacta of the substantia nigra (Fig. 1), the basolateral amygdala, and the central medial thalamic nucleus (not shown). Consistent with the block of anterograde transport in these neurons, BDNF immunoreactivity was markedly decreased in striatal neuropil and increased in white-matter fascicles within the corpus callosum and striatum, through which cortical neurons project to the striatum (Fig. 1). However, BDNF remained undetectable in striatal neurons after colchicine treatment.

To confirm that BDNF in the striatum was derived from extrinsic afferents, the frontoparietal cortex or nigrostriatal dopamine system was ablated unilaterally. In addition, the potential contribution of intrinsic neurons to striatal BDNF protein levels was evaluated

following the intrastriatal injection of quinolinic acid, an excitotoxin which destroys striatal neurons but spares the axons and terminals of striatal afferents. Quantitative changes in BDNF levels were determined by enzyme-linked immunosorbent assay (ELISA), and qualitative changes in BDNF distribution were evaluated immunohistochemically.

After unilateral cortical ablation, BDNF protein levels were decreased by 66% in the ipsilateral striatum and by 22% in the contralateral striatum (Fig. 2). The magnitude of these losses corresponds with the known contribution of cortical inputs to the striatum from the ipsilateral and contralateral hemispheres¹⁷. Unilateral injections of 6-hydroxydopamine into the medial forebrain bundle produced a 95% depletion of dopamine in the ipsilateral striatum and decreased BDNF protein levels by 14% compared with the contralateral, intact striatum ($P < 0.05$, paired t -test). This smaller decrease in BDNF is due to the small nigrostriatal dopamine input relative to the corticostriatal glutamatergic input. In contrast to lesions of cortical or nigral afferents to the striatum, the destruction of intrinsic striatal neurons with quinolinic acid did not decrease striatal BDNF levels (Fig. 2). This finding excludes the possibility that intrinsic striatal neurons contribute to BDNF protein levels in the striatum.

The loss of BDNF protein in the striatum after decortication, as determined by ELISA, was confirmed by a dramatic loss of BDNF immunostaining in the dorsal lateral striatum of the injured hemisphere (Fig. 3). A smaller decrease was apparent in the contralateral

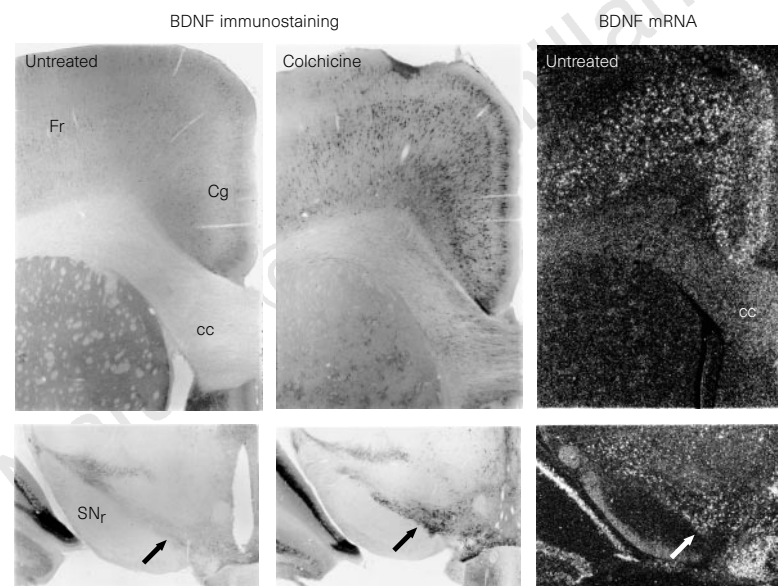


Figure 1 Top, BDNF staining in untreated rats reveals moderately intense immunoreactivity within the striatum (caudate-putamen, CPU), and the frontal (Fr) and cingulate (Cg) areas of cortex, but not in myelinated fibre tracts such as the corpus callosum (cc). After intraventricular colchicine treatment, BDNF immunoreactivity is markedly increased in cortical neurons and is reduced in the striatal neuropil. *In situ* hybridization reveals that neurons that express BDNF mRNA are distributed in the same pattern as those that exhibit BDNF staining following colchicine treatment. Bottom, In untreated rats, only light BDNF staining is found in the neuropil and a few cell bodies in the pars compacta of the substantia nigra (arrow). Following colchicine treatment, many neurons in the pars compacta (arrow) stain intensely for BDNF, and many similarly distributed neurons express BDNF mRNA.

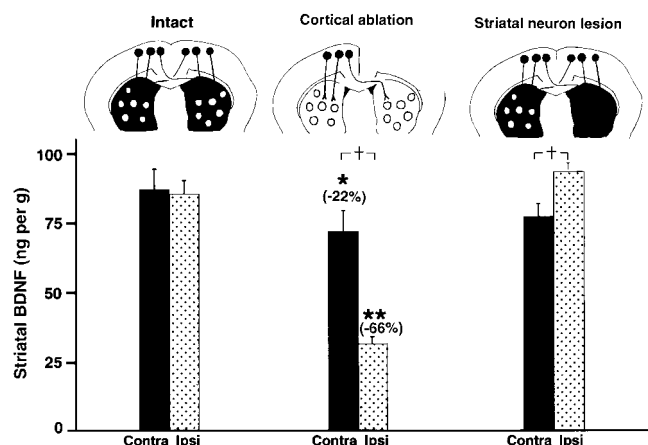


Figure 2 Striatal BDNF protein content in intact male rats ($n = 9$) or 1 week after an aspirative cortical ablation ($n = 6$) or an axon-sparing lesion of intrinsic striatal neurons with quinolinic acid ($n = 6$). Numbers above the bars represent the percentage decrease in BDNF content relative to the homolateral striata of intact animals. $*P < 0.02$ for the difference between the left striata of the decorticate and intact groups; $**P < 0.001$ for the differences between the right striata of the decorticate group and intact group, tests for contrast. $\dagger P < 0.01$ for the difference between the left and right striata, paired t -test. White and black circles in the coronal brain illustrations represent striatal and cortical neurons, respectively. Lines represent axon processes, and forked lines represent axon terminals. Shading represents the relative amounts of striatal BDNF remaining in each treatment group, with paler shading representing less BDNF. Contra, contralateral; Ipsi, ipsilateral.

striatum. Very dense staining of axonal processes, typically associated with large 'retraction bulbs', was prominent within the corpus callosum and subcortical white matter of the ipsilateral hemisphere (Fig. 3, inset). Presumably, this very dense staining indicates the accumulation of BDNF in the axons of corticocortical neurons that had their axons severed as they entered the aspirated neocortex.

The functional role of BDNF supplied to striatal neurons by cortical and other afferents was evaluated in mice that were heterozygous or homozygous for a null mutation in the gene encoding BDNF⁶. At 2 weeks of postnatal life these animals have no gross structural changes in the striatum, but the number of neurons that express the calbindin phenotype is substantially reduced⁴. Unlike calbindin, which is expressed in medium spiny projection neurons, parvalbumin is a marker of medium spiny GABAergic striatal interneurons that receive synaptic inputs from cortical neurons and do not project outside of the striatum^{18,19}. Parvalbumin immunostaining of brain sections from BDNF wild-type mice revealed numerous darkly stained neurons in the neocortex and striatum (Fig. 4, top left), with increasing neuron density and strong neuropil staining evident in the lateral striatum (Fig. 4, bottom left). In

contrast, BDNF^{-/-} mice showed many fewer parvalbumin-positive cells in the cortex (Fig. 4, top right), and decreased intensity and frequency of somatic and neuritic staining in the striatum (Fig. 4, bottom right). Both +/+ and -/- mice displayed a clear gene dose-related decrease in the average number of striatal neurons that expressed parvalbumin (Fig. 5). The numbers of parvalbumin-positive striatal neurons in the three genotype groups paralleled decreases in BDNF protein levels measured by ELISA¹⁵ in the hippocampus of wild-type (296 ± 17 ng BDNF per g tissue), +/- mice (147 ± 11 ng per g), and -/- mice (29 ± 5 ng per g).

Our results suggest that anterograde transport is important for the trafficking of BDNF in the CNS. Within the basal ganglia, this is demonstrated by several lines of evidence: substantial levels of BDNF protein are present in the striatum, despite there being a paucity of BDNF mRNA; striatal BDNF levels are unaffected by axon-sparing lesions of intrinsic neurons; BDNF protein and mRNA are present in striatal afferents but not striatal targets; inhibition of axonal transport depletes BDNF immunoreactivity in the striatum and increases it in the cortical and nigral neurons that project to the striatum; and lesions of corticostriatal and

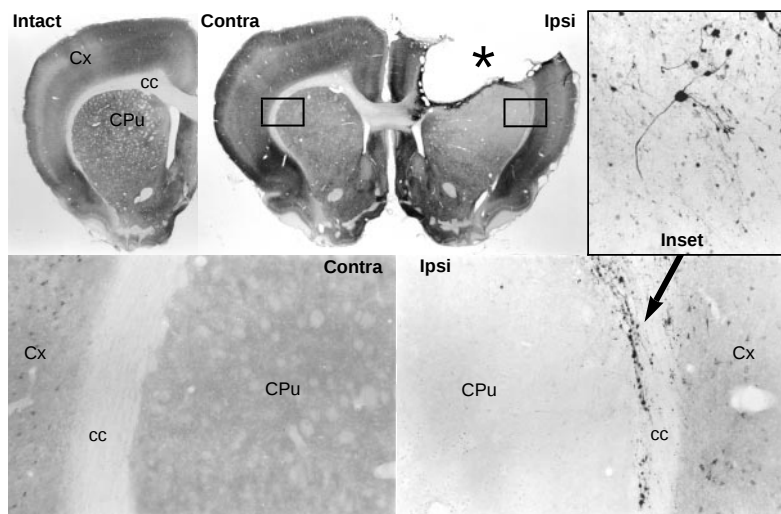


Figure 3 Top, BDNF immunostaining in an intact rat (left). A unilateral ablation of the frontoparietal cortex (right, asterisk) decreases BDNF immunostaining within the ipsilateral (Ipsi) striatum, and a smaller reduction is visible in the contralateral (Contra) striatum. Bottom, Photomicrographs correspond to the outlined dorsolateral striatum in the top right photomicrograph. Contralateral to the lesion, BDNF immunoreactivity is present in cortical and striatal neuropil and cortical neurons. BDNF staining in the striatal neuropil ipsilateral to the cortical ablation is reduced to a level comparable to that of the immunonegative fascicles of white matter. Intense BDNF staining occurs in axons and large, dilated profiles within the corpus callosum and subcortical white matter (arrow, and in the higher-magnification insert).

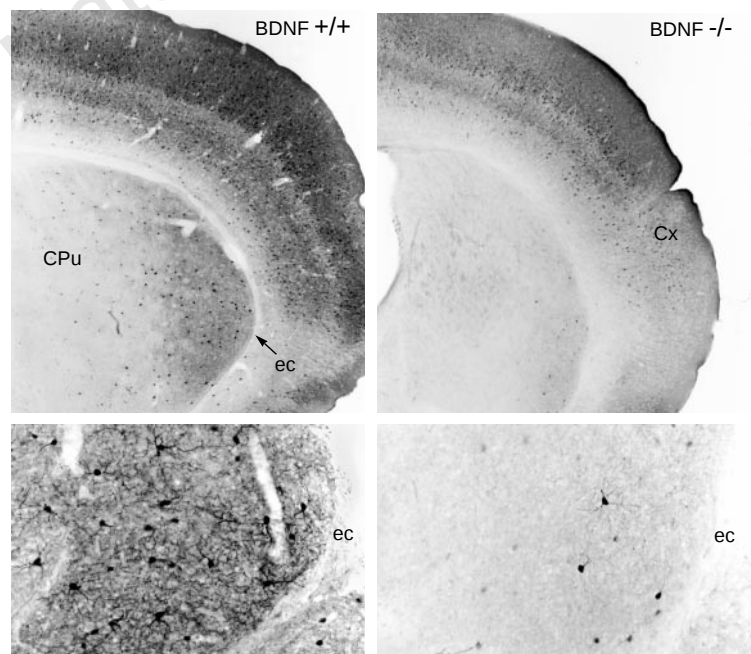


Figure 4 Top, Representative parvalbumin immunostaining in a wild-type mouse (BDNF +/+, left) and a homozygous deletion mouse (BDNF -/-, right). Bottom, Higher-power photomicrographs of the lateral striatum reveal numerous parvalbumin-positive neurons and a dense neuropil staining of a wild-type mouse (left), and less staining in the homozygous null mutant mouse (right). CPu, caudate putamen; Cx, Cerebral cortex; ec, external capsule.

nigrostriatal projection neurons deplete striatal BDNF. Thus the anterograde transport of BDNF by cortical and nigral neurons accounts for most of the BDNF in the adult rat striatum.

The presence of BDNF protein in the adult rodent striatum is consistent with its known actions on striatal neurons. BDNF binds with high affinity in the striatum, and mRNA for the BDNF receptor, TrkB, is found there^{20,21}. In cell culture, BDNF increases the survival of striatal GABA neurons, increases soma size, and promotes dendritic branching^{22,23}. Infusions of BDNF into the adult rat striatum alter the expression of neuropeptides and the GABA-synthetic enzyme, glutamic acid decarboxylase, and induce behavioural changes consistent with increased striatal output^{1,2,9}. The establishment of the calbindin⁴ and parvalbumin phenotype of striatal neurons depends on endogenous BDNF. As nearly all of the BDNF in the adult striatum is present in the terminals of afferent neurons, it is likely that the biological effectiveness of endogenous BDNF requires its terminal release. The recent identification of BDNF within synaptic fractions of brain homogenates²⁴ and the preferential localization of TrkB to postsynaptic densities²⁵ are consistent with BDNF being released from nerve terminals. Our demonstration of deficits in the complement of parvalbumin-immunopositive neurons in the striata of BDNF null mutant mice that correlate with the deficit of the BDNF gene and protein content indicate a clear functional role of anterogradely transported BDNF.

Our data and recent findings¹³ provide evidence that anterograde transport is important for the trafficking of an endogenous growth factor in the adult mammalian CNS. Anterograde transport of endogenous BDNF has also been observed in the peripheral nervous system²⁶. The anterograde transport of exogenous NT-3 has been demonstrated in the retinotectal pathway of the chick²⁷. Taken together, these observations suggest that anterograde transport may be a common feature of neurotrophin trafficking in the nervous system. Our data suggest that anterograde transport and terminal release may constitute an important means of neurotrophic support, much as neurons can derive trophic factors from their targets by way of retrograde transport. Loss of 'afferent supplied' trophic support may account for some of the degenerative changes that follow deafferentation, including somal and dendritic atrophy, decreases in neurotransmission, and neuron death. □

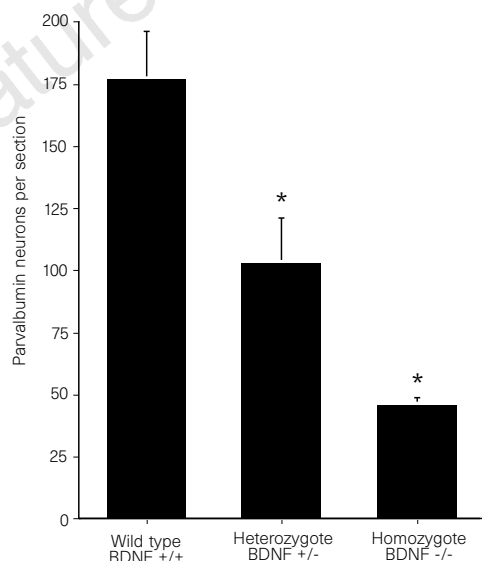


Figure 5 Parvalbumin-immunopositive neurons were counted in each striata of a single section taken at the level of the decussation of the anterior commissure in 15-day-old mouse pups: wild type ($n = 5$), +/- ($n = 6$) or -/- ($n = 5$). The average number of neurons per striatum $\pm$ s.e.m. differed as a function of genotype group ($F(2, 13) = 15$; $P < 0.001$) and significant differences were obtained between wild type and each of the knockout groups (* $P < 0.05$, Tukey-Kramer analysis).

Methods

Rats and surgical treatments. Male Sprague-Dawley rats (180–240 g) were housed and treated in compliance with AALAC guidelines²⁸. All animal-related procedures were approved by the institutional animal care and use committee. For stereotaxic procedures, animals were anaesthetized by intraperitoneal injection of chloral hydrate (130 mg per kg body weight) and sodium pentobarbital (26 mg per kg).

In situ hybridization histochemistry. BDNF mRNA was localized in fixed, free-floating sections as described^{7,8} using an 800-bp ³⁵S-cDNA probe.

Inhibition of axonal transport. Colchicine was injected with stereotaxic guidance into the lateral cerebral ventricles (50 μ g per side) and the animals allowed to survive for 36–48 h. Colchicine-treated and unoperated control animals were exsanguinated under deep anaesthesia and perfused transcardially with phosphate buffered saline followed by 2% paraformaldehyde and 0.2% parabenzoquinone. After brief postfixation, brains were cryoprotected in phosphate buffer containing 30% sucrose. BDNF immunostaining was performed on series of free-floating, coronal sections (40 μ m) using an affinity-purified rabbit polyclonal antibody generated against recombinant human BDNF (provided by Q. Yan and J. Miller, Amgen)¹⁴.

Cortical aspiration lesion. Each rat was mounted in a small animal stereotaxic apparatus (Kopf, Tinunga, CA). The top half to two-thirds of the exposed frontoparietal and cingulate cortices were removed by aspiration. This produced an ischaemic necrosis of the remaining deep cortical layers, but spared the underlying structures which receive a separate vascular supply. The cortical wound was filled with gel-foam and the cutaneous wound was closed with metal clips.

Nigrostriatal dopamine pathway lesion. The nigrostriatal dopamine pathway was ablated unilaterally by two injections of 8 μ g of 6-hydroxydopamine into the right substantia nigra and medial forebrain bundle. Animals were killed by decapitation 4 weeks later, and a circular punch of tissue 2 mm in diameter removed from the central striatum ipsilateral and contralateral to the lesion. HPLC analysis demonstrated that dopamine lesions were 95% complete. The remainder of the striatum was dissected and BDNF content was determined by ELISA.

Intrinsic striatal neuron lesions. These were produced with intrastriatal injections of 50 nmol of quinolinic acid. Animals were killed by decapitation 1 week later, and a 3 mm $\times$ 3 mm area of lesioned striatal tissue and similarly located tissue on the contralateral side were removed from a 1-mm slab.

BDNF ELISA. After the animals were killed by decapitation, the lateral two-thirds of each striatum was dissected rostral to the globus pallidus according to the topography of cortical inputs to the striatum¹⁹ and assayed for BDNF protein content¹⁵.

BDNF immunocytochemistry. Rats that had received unilateral cortical ablations and age-matched controls were deeply anaesthetized and perfused transcardially with heparinized saline followed by 4% phosphate-buffered paraformaldehyde, pH 7.0, containing 7.5% saturated picric acid (v/v). The brains were removed, blocked in the coronal plane and cryoprotected in phosphate buffer containing 7.5% picric acid and 30% sucrose. A series of 40- μ m frozen sections were stained for endogenous BDNF using a polyclonal rabbit anti-BDNF antibody as described^{13,14}. The antibody is highly specific for BDNF, exhibiting less than 0.5% cross-reactivity with related neurotrophins on western blots, and produces no staining in brain sections of animals which had received continuous infusions of recombinant human NGF, NT-3 or NT-4 for 12 days (7–12 μ g per day).

Parvalbumin immunocytochemistry. Mouse pups 15 days old (wild types, or heterozygous or homozygous BDNF null mutants) were deeply anaesthetized and perfused transcardially with heparinized saline followed by 4% phosphate-buffered paraformaldehyde, pH 7.2. The brains were placed in phosphate buffer containing 30% sucrose for 3–5 days. Coronal sections 40 μ m thick were stained for parvalbumin¹⁷.

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Metaplasticity at identified inhibitory synapses in *Aplysia*

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Synaptic plasticity is an important feature of neural networks involved in the encoding of information. In the analysis of long-term potentiation and long-term depression, several examples have emerged in which this plasticity is itself modulated^{1–3}. This higher-order form of plasticity has been referred to as ‘metaplasticity’⁴, a modification of synapses reflected as a change in the ability to induce or maintain plasticity. These observations raise the question of the possible advantage of regulating the intrinsic plastic properties of a synapse. The

neural circuit mediating the siphon withdrawal reflex in *Aplysia* provides a useful network in which to examine this question directly. Inhibitory synapses in this circuit (from L30 neurons) exhibit a variety of forms of activity-dependent short-term synaptic enhancement which contribute to dynamic gain control in the siphon withdrawal reflex^{5–9}. Here we report that tail shock, an extrinsic modulatory input of known behavioural relevance, induces differential metaplasticity at this synapse, attenuating its ability to exhibit short-term synaptic enhancement after pre-synaptic activation (augmentation and post-tetanic potentiation), while leaving intact its capacity for enhancement during activation. This attenuation of inhibition at the synaptic level seems to mediate comparable attenuation of inhibitory modulation at both network and behavioural levels.

The recurrent synaptic circuit in *Aplysia* between the L30 neurons and identified excitatory interneurons, the L29 neurons (Fig. 1Aa), provides activity-dependent regulation of excitatory input onto the LFS siphon motor neurons. Induction of short-term synaptic enhancement (STE) in L30 neurons dynamically increases the strength of their inhibitory input onto the L29 neurons during reflex activation, thereby reducing this source of excitatory input to the motor neurons⁵. STE in the L30 neurons can be induced by weak tactile stimulation of the siphon and tail, and by direct intracellular activation of L30 at rates comparable to those induced by tactile input (Fig. 1Ab). Conversely, strong input such as tail shock, a known facilitatory modulator of the siphon withdrawal reflex (SWR)^{10,11}, produces inhibition of the L30 neurons⁶. As in other systems^{7,12,13}, STE in L30 neurons can be divided into three components on the basis of the duration of expression: frequency facilitation is a short-lasting (<1 s) synaptic enhancement that is expressed during cell activation, whereas augmentation and post-tetanic potentiation (PTP) last longer (seconds to minutes) and are expressed primarily after cell activation¹⁴. Because augmentation and PTP are mechanistically similar^{7,12,15}, we will consider them together as a single (combined) component of STE.

Because of the regulatory role of the L29/L30 circuit and the modulatory role of tail shock, we were interested in examining how tail shock might affect STE at the synapse between L30 and L29 neurons, an analysis that can be readily performed using a reduced experimental preparation (Fig. 2Aa). STE was examined at the same synapse before and 90 s after shock (Fig. 1B). Two kinds of analysis were performed. First, the net synaptic current was examined. As reported previously¹⁰, tail shock significantly reduced basal synaptic transmission. We also found that the various components of STE were differentially affected by the shock: no difference in synaptic enhancement was observed during activation (frequency facilitation), but there was a significant attenuation in enhancement after activation (augmentation/PTP) (Fig. 1Bb). In the second analysis we took advantage of the fact that, both before and after the shock, at the end of activation (frequency facilitation) the synaptic currents are equivalent, despite baseline differences. We could therefore use the amplitude of the facilitated synapse at the end of activation (the average of the last five inhibitory postsynaptic currents) as a normalization point to analyse augmentation and PTP before and after tail shock. Taking the 20-s time point for comparison, significant augmentation/PTP was observed before shock (200%, $P < 0.01$), whereas no significant augmentation/PTP was observed after shock (39%, $P = 0.10$). Moreover, the difference between preshock and postshock measures was significant ($P < 0.01$). These results indicate that, at the same synapse, an extrinsic modulatory stimulus can have a long-lasting effect on the induction of one form of synaptic plasticity (augmentation/PTP) while sparing another (frequency facilitation). Subsequent examination of L30 STE at 10-min intervals for up to 40 min after shock indicated that both the reduction in the baseline synaptic current and in augmentation/PTP were maintained, with no concurrent change in frequency facilitation (data not shown).

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We have previously shown that augmentation/PTP in the L30 neurons leads to the inhibition of the siphon-evoked reflex input to siphon motor neurons observed for up to 60 s after weak tactile stimulation of the tail⁶. The observation that the induction of augmentation/PTP could be significantly attenuated by tail shock (Fig. 1Bb) led us to examine whether this suppression had any effect on network-level inhibitory regulation induced by tactile stimulation. The experimental design is shown in Fig. 2Ab. Two test phases were examined, phase 1 being before tail shock, and phase 2 after tail shock. In both phases, in control (no shock) preparations (Fig. 2Ba), tactile stimulation of the tail induced significant and repeatable inhibitory modulation of siphon-evoked responses in motor neurons (complex excitatory postsynaptic potential). However, in experimental preparations (Fig. 2Bb), after tail shock (phase 2), no significant inhibition was observed following tactile stimulation, indicating that tail shock abolished inhibitory modulation. Because

tail-shock stimulation was at a lower level in these experiments than in other studies¹¹, inhibitory modulation was abolished even in the absence of the induction of overt reflex facilitation (sensitization) in shocked preparations (Fig. 2c) (controls showed a significant reduction in phase 2, presumably resulting from a chance elevation in phase 1 scores, as depression is not normally observed under these test conditions^{5,6}). In summary, these data indicate that the same modulatory stimulus that suppresses augmentation/PTP in the L30 neurons at the synaptic level can also eliminate network-level inhibition induced by tactile stimulation. Because this form of network inhibition can be attributed directly to augmentation/PTP in the L30 neurons⁶, these results support the conclusion that the network effects of tail shock (Fig. 2) are due to a reduction in L30-mediated inhibition.

We then extended our analysis to intact, freely moving animals. We began by examining whether reflex inhibition could be induced

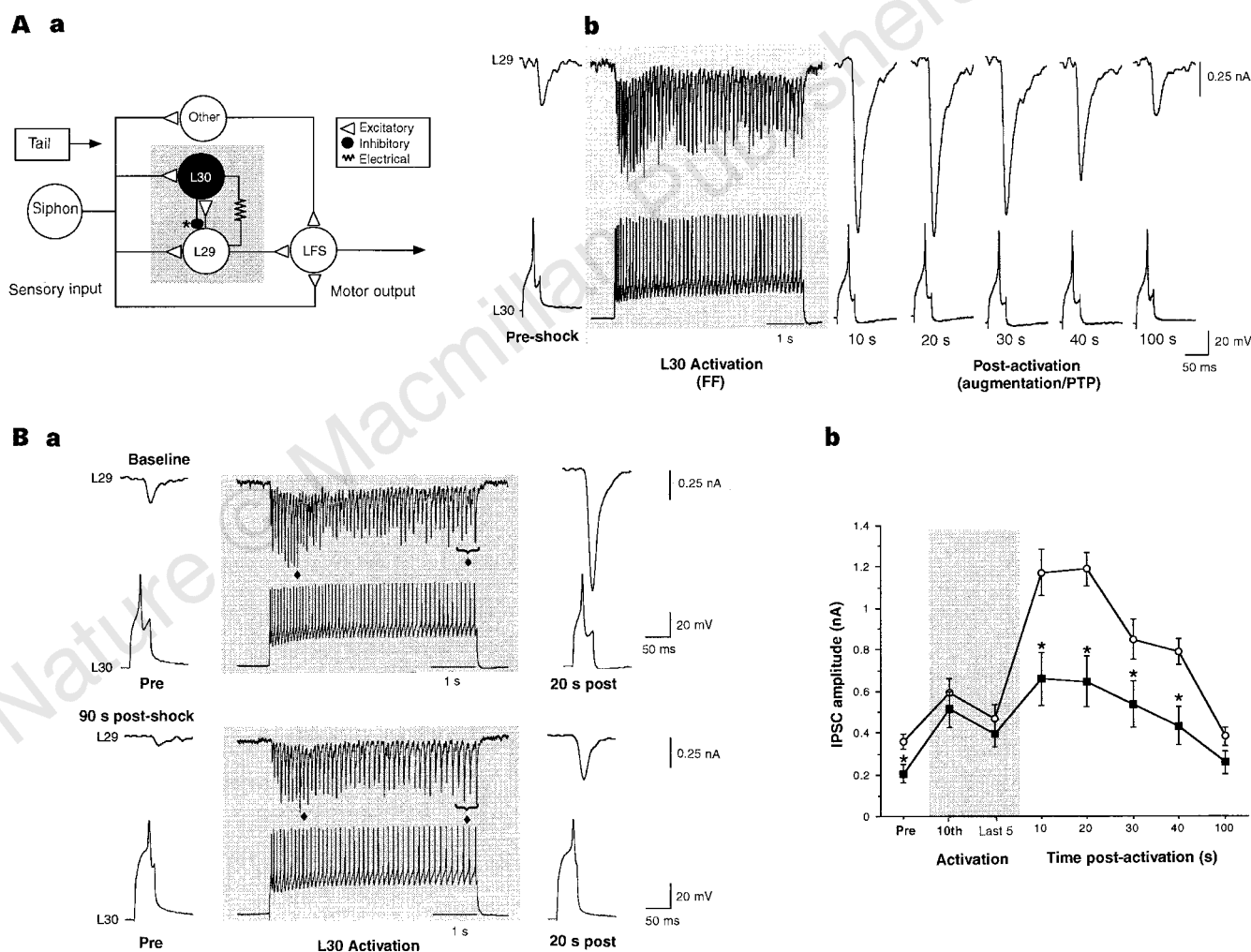


Figure 1 Tail shock suppresses specific components of L30 short-term synaptic enhancement (STE). **A, a**, Simplified schematic representation of the SWR circuit illustrating the synaptic relationship between the three L30 inhibitory interneurons and the five L29 excitatory interneurons. STE at the L30 inhibitory synapse (asterisk) regulates the amount of L29-mediated excitatory input onto LFS-type siphon motor neurons during reflex activation^{5,6}. L29 and L30 are both activated by weak tactile stimulation of the tail. The L30s are inhibited by noxious tail shock (which activates the L29s)^{6,28}. **b**, Simultaneous recordings from an L29 interneuron (top) and an L30 interneuron (bottom) illustrating the different components of L30 STE. The enhancement during activation (shaded) reflects frequency facilitation (FF), whereas enhancement following activation reflects combined augmenta-

tion and post-tetanic potentiation (augmentation/PTP). **B, a**, Examination of the same L30 synapse before (baseline, pre-shock) and 90 s after tail shock. In the baseline trial, typical FF (shaded) and AUG/PTP (shown at the 20 s test) are observed. After tail shock, the amplitudes of the L30 IPSC for both pre-shock and AUG/PTP measures are reduced, whereas measures during activation (FF) are unchanged. **b**, Data from 11 experiments examining L30 STE before (open circles) and 90 s after (filled squares) tail shock. FF measurement points are indicated by diamonds in the shaded areas of **a**. Significant differences between baseline and post-shock trials ($P < 0.05$; indicated by an asterisk) were observed for both pre-shock and AUG/PTP measures; however, no differences were observed for measures of FF.

by the same tactile stimulus that induces network inhibition (Fig. 2Ba), and if so, whether it would also be suppressed by tail shock (as in Fig. 2Bb). We examined the SWR 1 min after tactile stimulation (Fig. 3A), and found that tactile stimulation significantly decreased the duration of the reflex compared with both the stimulated group's own baseline measures and to non-stimulated controls. We next examined whether this form of reflex inhibition was modulated by tail shock. In control animals (no shock), we observed typical reflex inhibition after tactile stimulation. However, in animals that received shock, the inhibition after tactile stimulation was abolished, indeed we observed a trend towards increased reflex responsiveness after tactile stimulation. As in cellular experiments, when comparing baseline scores after shock with measures obtained before shock, the elimination of inhibition occurred in the absence of any enhancement in the reflex (sensitization) (Fig. 3C). Thus, at a behavioural level, as in cellular studies, tactile stimulation of the tail induces inhibitory modulation of the SWR that is

eliminated by the same intensity of tail shock that attenuates augmentation/PTP at the L30 synapse.

Because serotonin (5-HT) is known to mediate many of the effects of tail shock in *Aplysia* neurons^{16–20}, we performed cellular experiments to examine whether bath application of 5-HT would mimic the effects of shock in attenuating augmentation/PTP in L30 neurons (Fig. 4A). Like tail shock⁶, application of 5-HT produced an inhibitory response in L30 neurons (not shown). In analysing net synaptic currents, when we evaluated the same L30 synapse before and 20 min after a brief (2 min) exposure to 5-HT (10 μ M), we observed a reduction in preactivation values, confirming a previous report¹⁰. As with tail-shock experiments (Fig. 1), we also observed a significant suppression of augmentation/PTP, but no change in the amplitude of frequency facilitation. In the second analysis (normalizing control and 5-HT conditions to the facilitated synapse), before exposure to 5-HT L30 activation produced significant augmentation/PTP (71%, $P < 0.01$), whereas after 5-HT exposure

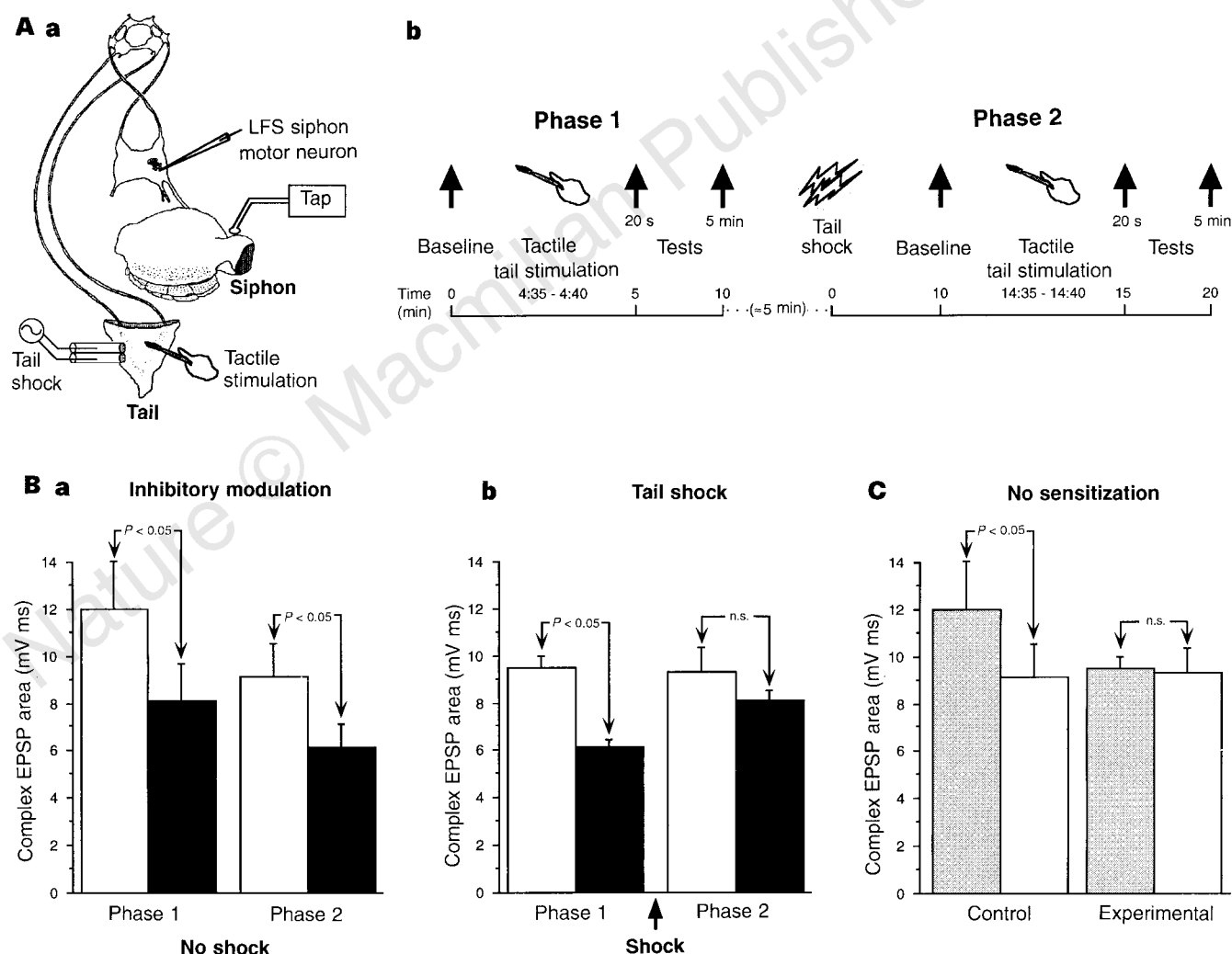


Figure 2 Tail shock suppresses L30-mediated network inhibition. **A, a**, Reduced experimental preparation. Reflex input (produced by brief tap to siphon) was measured as the complex EPSP in identified LFS-type motor neurons. Tactile stimulation of the tail was applied to a different site to the tail shock. **b**, Experimental design. Siphon taps are indicated by arrows. **B, a**, Quantitative summary from control (no shock) animals ($n = 6$). Baseline measures (white bars) and 20-s tests (black bars) are shown, for both phase 1 and phase 2. Significant reductions at the 20-s test were observed in both phases ($P < 0.05$), indicating that inhibitory modulation is repeatable. Recovery to baseline levels was observed at the 5 min test (not shown). **b**, Quantitative summary from experi-

mental (tail shock) animals ($n = 6$). Data are presented as in **a**. Typical inhibitory modulation was observed in phase 1 ($P < 0.05$). After tail shock (phase 2), test responses were not different from baseline levels, indicating that inhibitory modulation is significantly reduced. Five-min tests were not different from baseline levels. **C**, Baseline responses for control and experimental animals both before (grey, phase 1) and after (white, phase 2) tail shock. For experimental animals, no difference was observed, indicating that the mild tail shock used in these experiments did not induce overt sensitization. For controls, baseline responses were, by chance, significantly larger for phase 1 measures ($P < 0.05$).

augmentation/PTP was virtually abolished (synaptic strength decreased by 40%, $P = 0.06$). Moreover, the difference between 5-HT and control conditions was significant ($P < 0.01$). Thus application of 5-HT mimics the effects of tail shock (compare Fig. 3A with Fig. 1Bb). Although these results are consistent with many other findings implicating 5-HT in tail-shock effects^{16–20}, they do not demonstrate directly that 5-HT is responsible for the tail-shock effects we observe, nor do they exclude the participation of other known modulators in *Aplysia*²¹.

In a final series of experiments we examined the cellular mechanisms underlying the differential suppression of L30 STE by tail shock and 5-HT. Because the expression of STE is dependent on intracellular Ca^{2+} levels in the presynaptic terminal in L30 neurons¹⁴ (as in other systems^{7,15,22,23}), we examined the effects of reducing Ca^{2+} influx into L30 neurons during activation by systematically decreasing extracellular Ca^{2+} levels. In 25% Ca^{2+} (Fig. 4Ba), we observed a significant reduction in all measures. In 50% Ca^{2+} (Fig. 4Bb), as in tail-shock and 5-HT experiments, components of L30 STE were differentially affected: significant reductions occurred in both preactivation values and augmentation/PTP, but no change in frequency facilitation was observed. Moreover, when measures obtained 20 s post-activation were normalized to the facilitated synaptic current (frequency facilitation), augmentation/PTP was still significantly attenuated in 50% Ca^{2+} compared to control conditions ($P < 0.01$). Finally, in 75% Ca^{2+} (Fig. 4Bc), no significant changes in L30 STE were observed. These results indicate that frequency facilitation and augmentation/PTP have differential sensitivities to external Ca^{2+} (and presumably Ca^{2+} influx), with frequency facilitation requiring less Ca^{2+} for full expression than augmentation/PTP (Fig. 4C). Further, these results suggest that the effects of tail shock and 5-HT on L30 STE may be due at least in part

to a reduction in Ca^{2+} influx during activation to a level at which frequency facilitation is fully expressed, but below the threshold level required for the expression of augmentation/PTP.

In conclusion, we have shown that a specific modulatory form of metaplasticity can be induced by tail shock at identified synapses in *Aplysia*. We call this 'modulatory metaplasticity' because its induction does not require activity at the synapse whose plasticity is being regulated, as tail shock does not activate the L30 neurons⁶. But what are the functional consequences of this higher-order plasticity? By suppressing inhibitory plasticity in the SWR network, tail shock-induced metaplasticity at L30 synapses disinhibits the reflex system. This, in turn, could have widespread behavioural consequences, such as lowering the threshold for the subsequent induction of several forms of learning that involve an increase in reflex responsiveness (for example, dishabituation, sensitization and classical conditioning^{11,24}) by 'priming' the SWR network for facilitation. As the functional consequences of metaplasticity are identified in other systems, it will be interesting to see whether a comparable priming function may be a general feature of higher-order plasticity in neural circuits subserving learning and memory. □

Methods

Experimental preparations. Three types of experimental preparations were used: intact animals, reduced siphon/tail preparations, and isolated abdominal ganglia. In behavioural experiments using intact animals (Fig. 3), the parapodia were surgically removed to facilitate viewing of the siphon; surgery was performed following anaesthesia by immersion in ice-cold artificial sea water (ASW) composed of 460 mM NaCl, 55 mM MgCl_2 , 11 mM CaCl_2 , 10 mM KCl, 10 mM Tris, pH 7.4. Animals were allowed to recover for 3–5 days before behavioural testing. Reduced preparations⁶ (Fig. 2Aa) were used in cellular experiments examining the effects of tail shock (Figs 1, 2). For experiments

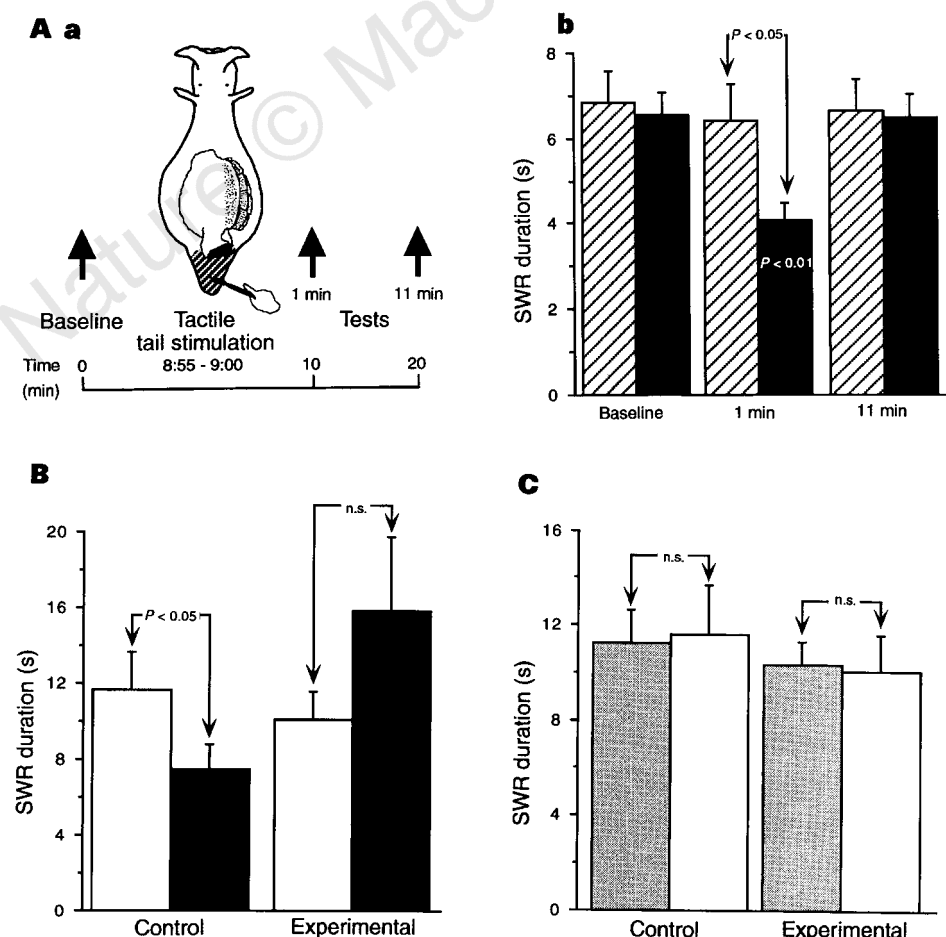


Figure 3 Tail shock suppresses inhibitory modulation in intact, freely behaving animals. **A**, **a**, Experimental design. In experiments examining the effects of tail shock, an additional measurement of the SWR was obtained before shock; baseline measures were then obtained 10 min after shock. **b**, Inhibitory modulation in intact animals. Experimental animals (black bars, $n = 18$) exhibited a reduced SWR duration at the 1-min test, which was significantly different from their own baseline scores ($P < 0.01$) and from control (hatched bars, $n = 18$) responses ($P < 0.05$). Reflex responses returned to baseline levels 10 min later. **B**, Summary of behavioural experiments examining the effects of tail shock on inhibitory modulation in intact animals. One min after tactile stimulation (black bars), a significant reduction in response compared with baseline (white bars) was observed in control animals ($n = 14$, $P < 0.05$), but not in experimental (shocked) animals ($n = 14$); indeed, a modest increase in responding was observed. **c**, SWR responses for experimental and control animals both before (pre-shock assessment, grey bars) and after tail shock (post-shock measures, white bars, same as baseline measures in **b**). For both groups, no significant difference was observed, indicating that overt sensitization was not induced by the level of tail shock used in these experiments.

examining the effects of 5-HT application and manipulations of extracellular Ca^{2+} (Fig. 4), the abdominal ganglion alone was placed in a small (4 ml) recording dish, which was perfused with ASW at 2 ml min^{-1} . For experiments examining the effects of bath-applied 5-HT, the perfusion solution was switched to one containing $10 \mu\text{M}$ 5-HT (Sigma) in ASW for 2 min (one complete bath exchange). This concentration was chosen because previous experiments showed that this (relatively low) level of 5-HT was sufficient to induce synaptic plasticity in *Aplysia* neurons²⁵. We then allowed 20 min for wash-out (~ 10 bath exchanges) to ensure the complete removal of 5-HT from the bath. For experiments in which the extracellular Ca^{2+} concentration was altered, the perfusion solution was switched to the reduced Ca^{2+} solution 5 min before physiological testing. Three different bath concentrations of Ca^{2+} were examined: 25% normal (2.75 mM), 50% normal (5.5 mM), and 75% normal (8.25 mM).

Experimental procedures: cellular analysis. Neurons were impaled with glass microelectrodes (resistance $10 \text{ M}\Omega$) containing either 3 M KCl or 0.6 M K_2SO_4 and 20 mM KCl. Two identified classes of interneurons, the L30s (presynaptic) and the L29s (postsynaptic), and the LFS siphon motor neurons were examined. The interneurons were identified by their recurrent synaptic relationship (Fig. 1Aa)^{5,26}. The magnitude of L30 synaptic strength was measured as inhibitory postsynaptic currents (IPSCs) in L29 interneurons voltage clamped at -85 mV , thus inverting the IPSC. STE in L30 neurons was induced by activating them directly for 5 s at 6–11 Hz. To capture the dynamics of enhancement during cell activation, we measured the 10th evoked IPSC (near maximum frequency facilitation) and obtained the average of the last 5 evoked responses (steady-state enhancement). Postactivation measures (aug-

mentation/PTP) were then obtained every 10 s after activation up to 100 s. Experiments examining modulation of the net reflex input to siphon motor neurons (Fig. 2) used the reduced preparation. LFS motor neurons were identified by their ability to evoke distinct siphon movements^{10,27}. Motor neurons were hyperpolarized to prevent action potentials, and siphon tap-evoked responses were measured as the area under the initial 500 ms of the complex EPSP^{5,6}. The test stimulus was a brief (50 ms) application of a mechanical tapper to the siphon, administered at a non-decrementing 5 min inter-stimulus interval. The tapper was positioned at the base of the siphon (attachment point to mantle), so that siphon movement did not result in a change in the site of stimulation. Animals were randomly assigned to either experimental (tail shock) or control groups, with the experimenter blind as to the group assignment. Experiments were performed in two phases (Fig. 2Ab). First, the baseline level of inhibition was assessed in all animals (phase 1) by recording the response to siphon tap before and 20 s after tactile stimulation of the tail (the magnitude of inhibition produced by tail stimulation is near maximum at this time point⁶). Stimulation was performed by gently brushing the tail with a no. 2 camel-hair paintbrush. Within 5 min of the last post-test, experimental animals received tail shock (1 s; 60 mA a.c.), applied using a bipolar electrode which spanned a surface area of 3 cm. Tactile stimulation-induced inhibition was assessed 10 min after shock (phase 2). Sensitization was assessed by comparing pretactile stimulation measures obtained in phases 1 and 2; sensitization would be reflected by an increase in reflex responsiveness after tail shock^{11,24}.

Behavioural testing. In all experiments, the SWR was elicited by brief application of a nylon bristle to the inner lumen of the siphon. The resulting

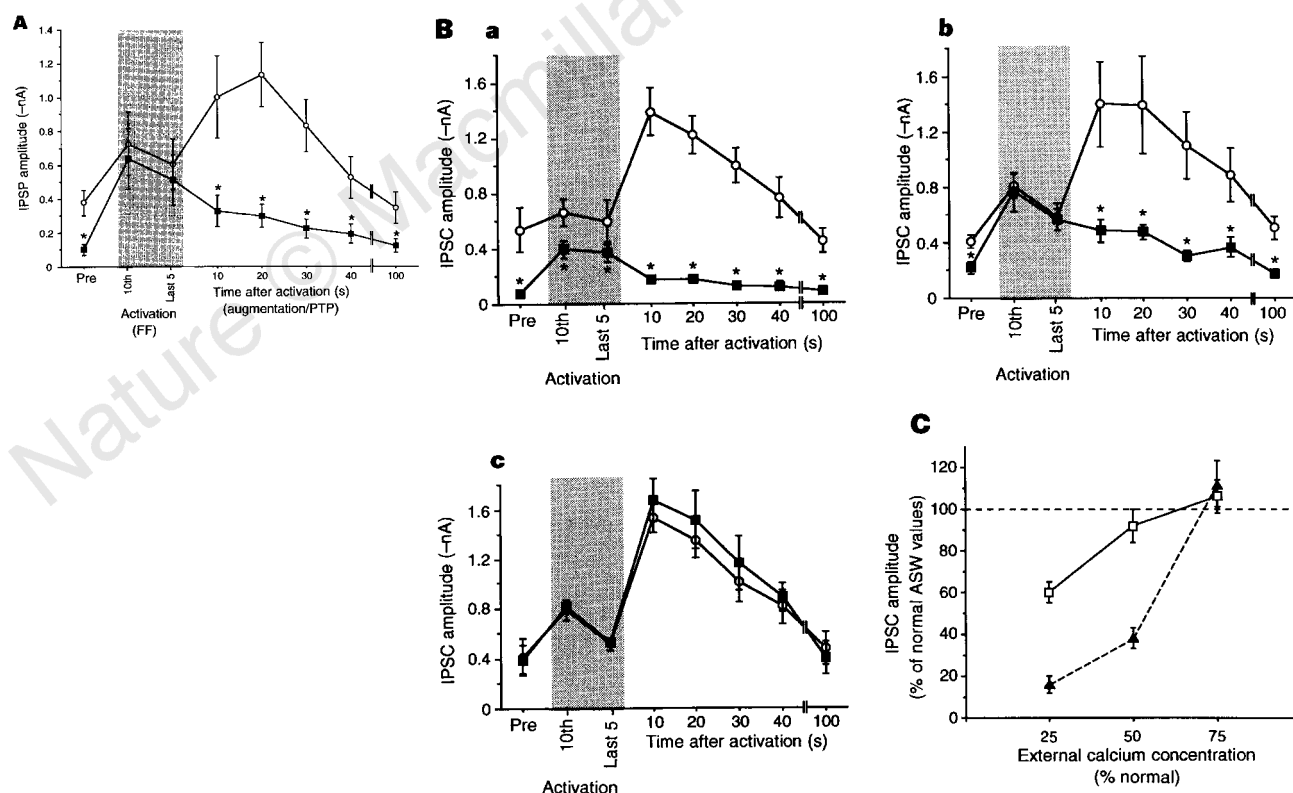


Figure 4 Possible cellular mechanisms of the suppression of augmentation/PTP in L30. **A**, Application of 5-HT mimics tail-shock effects on L30 STE ($n = 7$). As in tail shock experiments, 20 min after 5-HT treatment (filled squares), significant differences (asterisks) from baseline (open circles) measures were observed for pre-shock (Pre) and augmentation/PTP tests ($P < 0.05$), but not for measures of frequency facilitation (FF). **B**, Components of L30 STE exhibit differential sensitivity to external Ca^{2+} concentration. L30 STE was measured at the same synapse in normal ASW (open circles) and in one of three levels of reduced external Ca^{2+} concentration (filled squares). Significant differences from normal ASW measures of L30 STE are indicated by asterisks ($P < 0.05$). **a**, In 25% normal Ca^{2+}

($n = 5$), baseline synaptic transmission and both measures of L30 STE are reduced. **b**, In 50% normal Ca^{2+} ($n = 5$), significant differences were observed for pre and augmentation/PTP measures, but not for measures of FF (compare Figs 1Bb, 3A). **c**, In 75% normal Ca^{2+} ($n = 5$), all measures were the same as in normal ASW. **C**, Summary of experiments examining the effects of reducing external calcium concentration. FF (measured as the 10th IPSC in the spike train, open squares) and augmentation/PTP (measured 20 s after activation, filled triangles) are expressed as a function of different levels of calcium concentration, and are normalized to their respective baselines obtained in normal ASW. FF and augmentation/PTP exhibit differential sensitivities to external Ca^{2+} levels.

response was scored as the time from the onset of the withdrawal to the onset of relaxation of the siphon¹¹ by an observer blind to the group assignment of the animal. Siphon stimuli were delivered at a non-decrementing ISI of 10 min. Tactile stimulation of the tail was performed as in cellular experiments; post-tests were given 1 and 10 min after stimulation (a 1-min post-test was used to allow for full recovery of the behavioural response to tail stimulation). To examine the effect of tail shock on tactile stimulation-induced inhibition of the SWR, we first assessed the baseline response of the SWR. Animals were then assigned to experimental (tail shock) or control (no shock) groups to equate baseline response duration. Tail-shock parameters were the same as in cellular experiments. Inhibitory modulation was then characterized 10 min after tail shock. Sensitization was assessed by comparing measures obtained before and 10 min after shock.

Statistics. All summary data are presented as means of raw scores; error bars indicate s.e.m. Cellular data examining L30 synaptic enhancement were analysed using analysis of variance (ANOVA). In behavioural and motor-neuron experiments, comparisons of test responses to baseline responses were analysed using a *t*-test for related means. All probability values are two-tailed.

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Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene

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Excitatory amino acids induce both acute membrane depolarization and latent cellular toxicity, which often leads to apoptosis in many neurological disorders^{1,2}. Recent studies indicate that glutamate toxicity may involve the c-Jun amino-terminal kinase (JNK) group of mitogen-activated protein (MAP) kinases^{3–5}. One member of the JNK family, Jnk3, may be required for stress-induced neuronal apoptosis, as it is selectively expressed in the nervous system^{6,7}. Here we report that disruption of the gene encoding Jnk3 in mice caused the mice to be resistant to the excitotoxic glutamate-receptor agonist kainic acid: they showed a reduction in seizure activity and hippocampal neuron apoptosis was prevented. Although application of kainic acid imposed the same level of noxious stress, the phosphorylation of c-Jun and the transcriptional activity of the AP-1 transcription factor complex were markedly reduced in the mutant mice. These data indicate that the observed neuroprotection is due to the extinction of a Jnk3-mediated signalling pathway, which is an important component in the pathogenesis of glutamate neurotoxicity.

The JNK group of protein kinases (also known as SAPK) phosphorylates the amino-terminal activation domain of the transcription factor c-Jun^{8–10}. JNK is activated by MKK4 (also known as Sek1) and MKK7 in response to environmental stress and mitogenic factors^{11–15}. In addition to c-Jun, JNK also phosphorylates ATF2 and other Jun-family proteins that function as components of the AP-1 transcription factor complex^{7–10,16}. The phosphorylation of these transcriptional factors by JNK leads to increased AP-1 activity¹⁰. Transfection studies using constitutively activated and dominant-negative components of the JNK signalling pathway established that JNK was critically involved in the apoptosis of NGF-deprived PC12 cells⁵, a model of neuronal death *in vitro*.

A major challenge to understanding the function of JNK *in vivo* is imposed by the complexity of this gene family. Ten JNK isoforms resulting from alternative splicing of three genes have been identified⁷. Although Jnk1 and Jnk2 are widely expressed in murine tissues, including the nervous system (data not shown), the Jnk3 isoform is selectively expressed in the brain and, to a lesser extent, in the heart and testis^{6,7}. This restricted expression, together with the activation by noxious stimuli, suggests that Jnk3 may mediate stress-induced neuronal apoptosis. To test this hypothesis, we used gene targeting to generate Jnk3-deficient mice, and examined their response to noxious stimuli *in vivo*.

We designed a targeting vector to replace an internal 4-kilobase (kb) MscI–SpeI Jnk3 genomic fragment with a PGKneo cassette (Fig. 1a). The deleted region encompasses one and a half exons

encoding amino acids 211–267 of Jnk3. This region includes the tripeptide dual phosphorylation motif Thr-Pro-Tyr, which is characteristic of the JNK group and is required for protein kinase activity⁹. The linearized targeting vector was electroporated into W9.5 embryonic stem (ES) cells. Southern blot analysis of 104 independent G418- and gancyclovir-resistant clones revealed three clones containing the desired homologous recombination event (targeting frequency 2.9%). Chimaeric mice were generated by injecting these ES cells into C57BL/6 (B6) blastocysts. Two clones resulted in germline transmission of the disrupted *Jnk3* allele. Heterozygotes (+/-) were intercrossed to generate homozygous mutant mice (-/-) that were identified by Southern blot analysis of genomic DNA (Fig. 1b). Northern blot analysis using a *Jnk3* cDNA probe detected transcripts in the total brain RNA of wild-type (+/+) mice

but not homozygous knockout (-/-) mice (Fig. 1c). Reverse transcription-polymerase chain reaction (RT-PCR) analysis also confirmed the absence of *Jnk3* transcripts in the homozygous *Jnk3* (-/-) mouse brain (data not shown). Moreover, protein kinase assays demonstrated that the *Jnk3* (-/-) mouse hippocampus was deficient in JNK3 activity (Fig. 1d). Together, these data demonstrate that the targeted disruption of the *Jnk3* gene resulted in a null allele. The *Jnk3* (-/-) mice were fertile and of normal size. Histological surveys of a variety of tissues revealed no apparent abnormality. The *Jnk3* (-/-) mouse brain was examined by Nissl's stain and by immunocytochemistry using neuronal and glial cell markers, and had normal structural organization and cellular composition (data not shown). Moreover, a comparable number of motor neurons in the facial nucleus were found in wild-type and *Jnk3* (-/-) mice

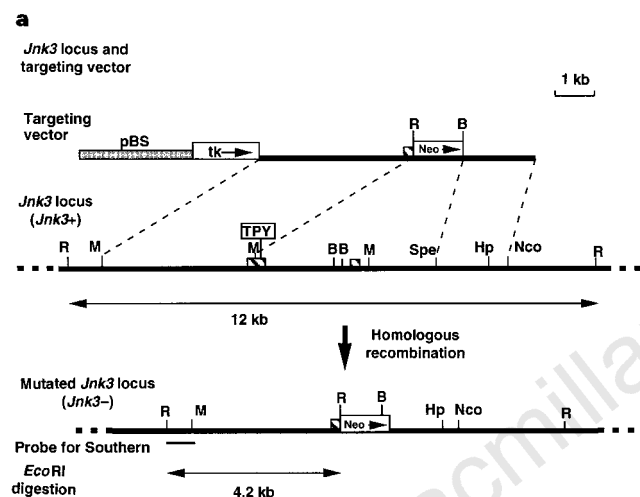
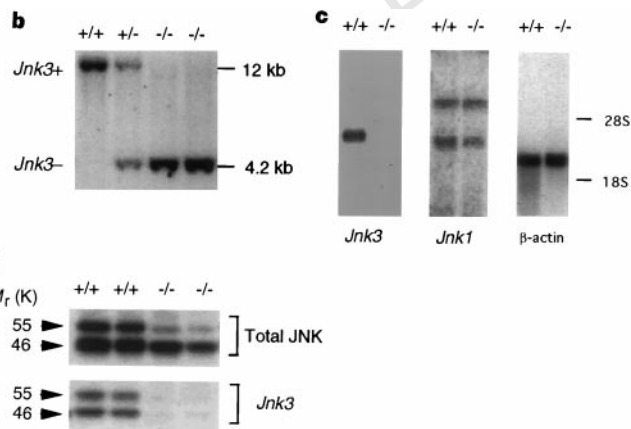


Figure 1 Generation of *Jnk3*-deficient mice by homologous recombination. **a**, Schematic diagrams of the *Jnk3* targeting vector, the genomic region at the *Jnk3* locus, and the predicted structure of the mutated *Jnk3* gene. Restriction enzyme sites are indicated (B, *Bam*HI; Hp, *Hpa*I; M, *Msc*I; Nco, *Nco*I; R, *Eco*RI; Spe, *Spe*I). The two hatched boxes are *Jnk3* exons corresponding to subdomains VIII and IX (amino-acid residues 189–267). **b**, Southern blot analysis of genomic DNA identifies mice corresponding to all three expected genotypes. *Eco*RI-restricted DNA was probed with a radiolabelled fragment of *Jnk3*. The upper band (12 kb)



corresponds to the wild-type allele, and the lower band (4.2 kb) to the mutant allele. **c**, Northern blot analysis of total RNA (10 µg) isolated from mouse brain. The blot was probed with a random-primed ³²P-labelled mouse *Jnk3* cDNA probe, then stripped and sequentially reprobed with mouse *Jnk1* and β-actin cDNA probes. **d**, JNK activity *in vitro*. Mouse hippocampal lysates (30 µg) from wild-type (+/+) and homozygous knockout (-/-) brains were measured by in-gel protein kinase assays using GST-c-Jun as the substrate to detect the total JNK and *Jnk3* protein kinase activity.

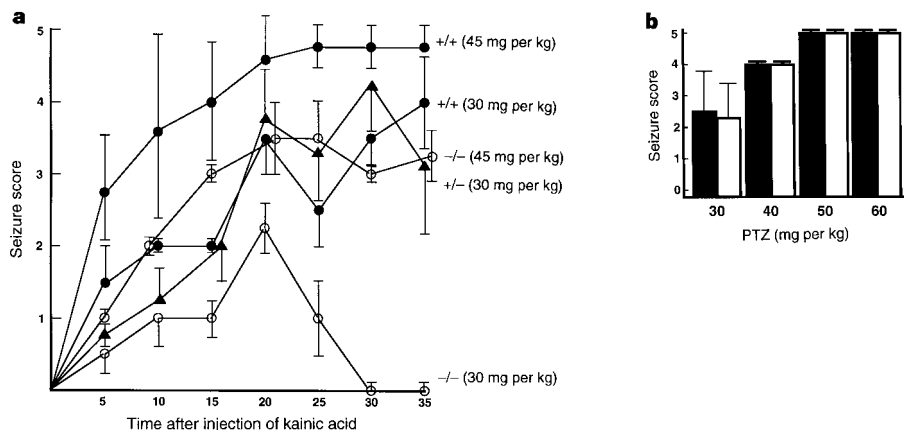


Figure 2 Comparison of seizure responses in littermates of varied *Jnk3* genotype. **a**, Seizure responses of adult wild-type (filled circles), *Jnk3* (+/-) (filled triangles) and *Jnk3* (-/-) (open circles) mice to intraperitoneal injection of kainic acid (30 or 45 mg per kg, as indicated). Seizures were scored³⁰ as follows: 1, arrest of motion; 2, myoclonic jerks of the head and neck, with brief twitching movements; 3, unilateral clonic activity; 4, bilateral forelimb tonic and clonic activity; 5, generalized tonic-clonic activity with loss of postural tone including death from contin-

uous convulsions. At least four mice in each group were observed and scored to derive the temporal response curve. **b**, Comparison of dose-response to pentetrazole (PTZ) between wild-type (black bars) and *Jnk3* (-/-) (white bars) mice. PTZ provoked rapid and abrupt general tonic-clonic convulsions. The same scoring criteria were used to record the PTZ-induced seizures within 5 min of injection (3 mice in each group).

(2,150–2,300 neurons per nucleus at postnatal day 10, $n = 4$). Thus there was no apparent developmental abnormality, including cell death, in the *Jnk3*($-/-$) mice.

The phenotype of *Jnk3* deficiency became apparent when mice were injected intraperitoneally with an epileptogenic dose of kainic acid, which elicits seizures by direct stimulation of glutamate receptors and indirectly by increasing the release of excitatory amino acids from nerve terminals^{17,18}. Unlike other epileptogenic agents, kainic acid when administered systemically causes neuronal damage, the most vulnerable region being the hippocampus¹⁷. The neurotoxicity of kainic acid has been suggested to result from potent induction of c-Jun and sustained AP-1 DNA-binding activity^{19,20}. Moreover, it has recently been demonstrated that kainic acid and glutamate induce JNK activity in cultured neurons^{3,4}. Together, these properties make systemic administration of kainic acid an appropriate model in which to study the function of *Jnk3* *in vivo*. We found that the administration of 30 mg of kainic acid per kg body weight induced seizures of progressive severity in both wild-type and heterozygous *Jnk3*($+/-$) mice (Fig. 2a). Mice first exhibited 'staring' spells, with abnormal body posture, progressed to head nodding ('wet-dog shakes'), forepaw tremor, rearing, loss of postural control, and eventually continuous convulsions. The kainic acid-induced seizures typically subsided 1 h after injection. In contrast, *Jnk3*($-/-$) mice showed much milder symptoms, mainly consisting of 'staring' spells and occasional myoclonic tremors, and recovered more rapidly. *Jnk3*($-/-$) mice developed seizures of comparable severity at a higher dosage of kainic acid (45 mg per kg, injected i.p.). However, at such high dosage, more than 60% of wild-type mice died from continuous tonic-clonic convulsions, whereas *Jnk3*($-/-$) mice survived (Fig. 2a).

Because a clear difference in susceptibility to kainic acid-induced seizures was observed among $+/+$, $+/-$ and $-/-$ littermates of the (B6 $\times$ 129) F₂ background, the resistance to kainic acid cannot be attributed to a difference in mouse strain. *Jnk3*($-/-$) mice were not resistant to pentetrazole (PTZ), another epileptogenic agent which induced seizures by blocking the GABA-inhibitory postsynaptic potential (IPSP)²¹. At all tested doses of PTZ (30, 40, 50 and 60 mg per kg, i.p.), *Jnk3*($-/-$) mice and wild-type littermates developed seizures of comparable severity (Fig. 2b). Moreover, unlike the slow progression of motor symptoms observed in the kainic acid-induced seizures, PTZ induced abrupt general tonic-clonic seizures within 5 min of injection, presumably resulting from an epileptogenic mechanism mediated by extracellular inhibition of GABA-IPSPs. Thus, neither poor drug delivery to the brain nor potent GABA-IPSPs in the neural circuit were likely to account for the lower susceptibility of *Jnk3*($-/-$) mice to kainic acid toxicity. We also examined the expression of the kainate-type receptor subunits GluR5–7, the NMDAR1 subunit, and the intracellular calcium-binding proteins calbindin and parvalbumin; no difference between *Jnk3*($-/-$) and wild-type mice was detected (data not shown). Together, these data indicate that the disruption of the *Jnk3* gene resulted in a selective resistance to kainic acid-induced seizures that cannot be explained by any apparent structural abnormality.

One possible explanation for the differential susceptibility of *Jnk3*($-/-$) mice to kainic acid is that *Jnk3* may be an important intracellular mediator of kainic acid excitotoxicity. To test this hypothesis, we first examined the kainic acid-induced expression of *c-fos* and *c-jun* to investigate whether an equivalent level of noxious stimulation was imposed on wild-type and *Jnk3*($-/-$) mice^{20,21}. Total RNA was extracted from the hippocampi of mice killed before and at 0.5, 2, 4 or 8 h after injection of kainic acid (30 mg per kg, i.p.). Northern blots were probed with murine *c-fos* and *c-jun* probes. *Jnk3*($-/-$) and wild-type mice exhibited a similar level of rapid induction of *c-fos* and *c-jun* transcripts that declined gradually 4 h after injection (Fig. 3a). We next compared the distribution of kainic acid-induced c-Fos and c-Jun immunoreactivity along the synaptic circuit of the hippocampus (Fig. 3b). In the

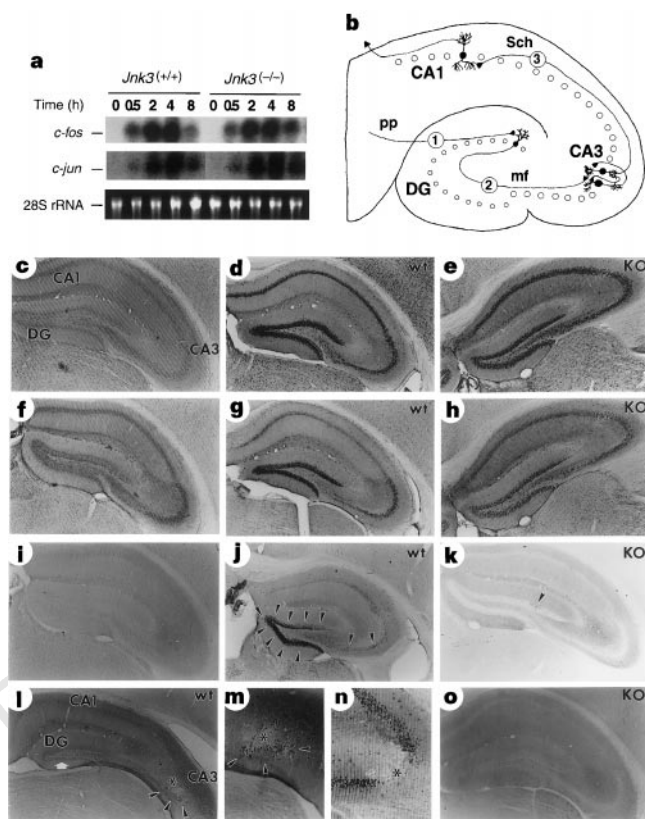


Figure 3 Comparison of kainic acid-induced expression of c-Fos, c-Jun and phosphorylated c-Jun in wild-type and *Jnk3*($-/-$) mice. **a**, Mice were killed at the indicated times after intraperitoneal injection of kainic acid (30 mg per kg). Hippocampal total RNA (two mice per group) was isolated from wild-type and *Jnk3*($-/-$) mice and probed with a murine *c-fos* cDNA probe, and subsequently stripped and re-probed with murine *c-jun* cDNA probe. RNA loading and transfer efficiency was monitored by ethidium bromide staining of 28S ribosomal RNA. **b**, Diagram of the tri-synaptic connection within the hippocampal formation. The first synaptic relay is from the afferent perforant path (pp) onto the granule cell of the dentate gyrus (DG). The second relay follows the mossy fibre (mf) from the dentate gyrus to the CA3 hippocampal neurons. The third relay is from the hippocampal CA3 to the CA1 region along the Schaffer collaterals (Sch). Note the recurrent synaptic interactions of pyramidal neurons within the CA3 region. **c–e**, Immunocytochemical analysis of c-Fos. The expression of c-Fos was greatly increased 2 h after injection of kainic acid (30 mg per kg, i.p.) from the untreated state (**c**) over the entire hippocampal region in both wild-type (**d**) and *Jnk3*($-/-$) (**e**) mice. **f–h**, Immunocytochemical analysis of c-Jun. The induction of c-Jun (**f**, untreated state) was localized to the dentate gyrus and the hippocampal CA3/CA4 region 2 h after injection of kainic acid in both wild-type (**g**) and *Jnk3*($-/-$) (**h**) mice. The magnitude of c-Jun induction was slightly greater in the wild-type mice. **i–m, o**, Immunocytochemical analysis of Ser 73 phosphorylated c-Jun. The expression of phosphorylated c-Jun was greatly increased in the dentate gyrus and the hippocampal CA3/CA4 region (arrowheads in **j**) 2 h after injection of kainic acid compared with the untreated state (**i**). In contrast, there was only minimal induction of phosphorylated c-Jun in the *Jnk3*($-/-$) mouse (**k**). At 6 h after injection of kainic acid, the expression of phosphorylated c-Jun in wild-type mice was restricted to the hippocampal CA3 region (arrowheads in **l**). Under higher magnification, a focus of damage (asterisk) in the CA3 region was enclosed by cells expressing phosphorylated c-Jun (arrowheads in **m**). The cell loss in the CA3 region is more evident in the adjacent section stained by c-Fos (**n**). In contrast, the expression of phosphorylated c-Jun was not found in the *Jnk3*($-/-$) mouse 6 h after injection of kainic acid (**o**). The ripple-like uneven surfaces are artefacts of the vibratome section procedure. KO, knockout; wt, wild type. Magnification: **c–l, o**, $\times 50$; **m, n**, $\times 200$.

absence of kainic acid there was no detectable c-Fos expression (Fig. 3c) and only a few c-Jun-positive cells within the dentate gyrus (Fig. 3f). By 2 h after injection of kainic acid (30 mg per kg, i.p.), there was a large increase in c-Fos immunoreactivity throughout the hippocampal region of both wild-type and *Jnk3*($-/-$) mice (Fig. 3d, e). There was a simultaneous increase in c-Jun-positive cells in the dentate gyrus and the CA3 region of the hippocampus of both wild-type and *Jnk3*($-/-$) mice (Fig. 3g, h). At 6 h after injection of kainic acid, the expression of c-Jun extended to the CA1 region in both wild-type and *Jnk3*($-/-$) mice (data not shown). The rapid induction of c-Fos and the more protracted expression of c-Jun is consistent with previous reports²¹. Because the induction of c-Fos and c-Jun is an indicator of neuronal activity following noxious stimulation²¹, the observation of similar levels of induction, time course and distribution of c-Jun- and c-Fos-labelled cells suggests that *Jnk3*($-/-$) and wild-type mice were subjected to an equivalent level of noxious stress by the systemic administration of kainic acid.

The activation of c-Jun is mediated by JNK through the phosphorylation of the N-terminal domain¹⁰. We examined the expression of phosphorylated c-Jun by using an antibody raised against c-Jun phosphorylated at Ser 73, one of the sites phosphorylated by JNK. Before kainic acid was administered, no cells were labelled by the antibody, in either wild-type (Fig. 3i) or *Jnk3*($-/-$) mice (data not shown). By 2 h after injection of kainic acid there was a high level of phosphorylated c-Jun in the dentate gyrus and the CA3/CA4 region of the hippocampus in the wild-type mice (arrowheads in Fig. 3j). In contrast, only a trace amount of phosphorylated c-Jun was detected in the *Jnk3*($-/-$) mice (arrow in Fig. 3k). Thus there was either a decreased level or less sustained phosphorylation of c-Jun in the *Jnk3*($-/-$) mice. There was also a dynamic change of the distribution of phosphorylated c-Jun in the wild-type mouse hippocampus. By 6 h after injection, the expression of phosphorylated c-Jun subsided in the dentate gyrus and progressed to a restricted area in the hippocampal CA3 region (arrows in Fig. 3l). Under higher magnification it was apparent that the expression of phosphorylated c-Jun surrounded an area of cell destruction (asterisk in Fig. 3m, n). In contrast, no labelling of phosphorylated c-Jun was detected in the *Jnk3*($-/-$) mice at the same time point (Fig. 3o). The hippocampal CA3 region is well documented as the structure that is most vulnerable to kainic-acid excitotoxicity, presumably as a result of both high levels of kainic-acid binding²² and potent excitatory synaptic connections between CA3 pyramidal neurons²³. The same

cellular and synaptic mechanism may contribute to the protracted phosphorylation of c-Jun in the hippocampal CA3 region.

The decreased phosphorylation of c-Jun in *Jnk3*($-/-$) mice may lead to a reduction of c-Jun/AP-1 transcriptional activity. To test this possibility, we crossed *Jnk3*($-/-$) mice with AP-1-luciferase (AP1-luc) transgenic mice to examine the kainic acid-induced AP-1 transcriptional activity. The AP1-luc mice contain the firefly luciferase reporter gene under the control of four copies of a consensus AP-1 binding site in the context of the minimal rat prolactin promoter²⁴. The expression level of luciferase in tissue extracts thus provides an assessment of AP-1 transcriptional activity *in vivo*. Control experiments demonstrated that the injection of vehicle (saline) alone did not cause induction of AP-1 transcriptional activity in the AP1-luc mice (data not shown). The injection of kainic acid (30 mg per kg, i.p.) caused a large induction of AP-1 transcriptional activity in the hippocampus of wild-type mice. The induced AP-1 transcriptional activity became detectable by 6 h, gradually increased to a peak at 3 days, and persisted for at least 7 days (Fig. 4, inset). In contrast, the induction of AP-1 activity was significantly reduced in the *Jnk3*($-/-$) mice. At 15 h after injection of kainic acid there was approximately fourfold greater AP-1 activity in the hippocampus of wild-type mice than *Jnk3*($-/-$) mice. At 3 days after injection, the AP-1 activity was more than six times higher in the hippocampus of wild-type mice (Fig. 4). The induction of AP-1 activity was more prominent in the hippocampus than the cerebellum and the cerebral cortex, consistent with the highest induction of phosphorylation of c-Jun being in this region. Together, these data demonstrate that disruption of the *Jnk3* gene suppressed kainic acid-induced phosphorylation of c-Jun and AP-1 transcriptional activity in the hippocampus *in vivo*.

It has been suggested that neuronal death induced by kainic acid is mediated by a sustained level of AP-1 transcriptional activity. We therefore examined whether the attenuation of AP-1 transcriptional activity in *Jnk3*($-/-$) mice would provide neuronal protection from kainic-acid cytotoxicity. Under Nissl's stain, the kainic acid-induced cell destruction caused either a diffuse sparse staining throughout the CA1 subfield (Fig. 5b) or a breach of staining of the CA3 hippocampal neurons (Fig. 5c). Under TUNEL staining, which detected DNA fragmentation in the dying cells, numerous strongly labelled cells were located in the hippocampal CA1 subfield of decreased Nissl's staining (Fig. 5e). Similarly, a group of TUNEL-positive cells were found in the hippocampal CA3 subfield devoid of Nissl's staining (Fig. 5f). We have also used immunostaining for damage-induced glial fibrillary acidic protein (GFAP) as an independent assessment of cell destruction. Consistent with the patterns of Nissl's and TUNEL staining, an increased number of strongly GFAP-labelled astrocytes were found either in the hippocampal CA1 (Fig. 5h) or CA3 (Fig. 5i) regions.

A total of 17 wild-type (+/+), 8 *Jnk3*(+/-) and 18 *Jnk3*($-/-$) littermates were examined for hippocampal damage 5 days after systemic administration of 30 mg per kg kainic acid. Prominent cell destruction was found in a large proportion of wild-type (13 of 17, 75%) and heterozygous mice (6 of 8, 75%). Consistent with previous reports, the CA3 subfield appeared to be the most vulnerable hippocampal region in both wild-type (9 of 13, 69%) and heterozygous mice (6 of 6, 100%). In contrast, none of the 18 *Jnk3*($-/-$) mice showed signs of cell damage despite equivalent expression of *c-fos* and *c-jun* in these littermates. The Nissl's stain (Fig. 5a), TUNEL labelling (Fig. 5d) and GFAP-immunostaining (Fig. 5g) of *Jnk3*($-/-$) mice that received 30 mg per kg kainic acid all showed an indistinguishable pattern from the untreated wild-type mice. Moreover, although *Jnk3*($-/-$) mice developed seizures of comparable severity at the sublethal dose of 45 mg per kg kainic acid (a dose that is lethal for >60% of wild-type mice as a result of continuous convulsions), cell damage was nevertheless found in a much smaller proportion of animals (2 of 15, 13%; $P < 0.005$ by chi-square analysis, d.f. = 1).

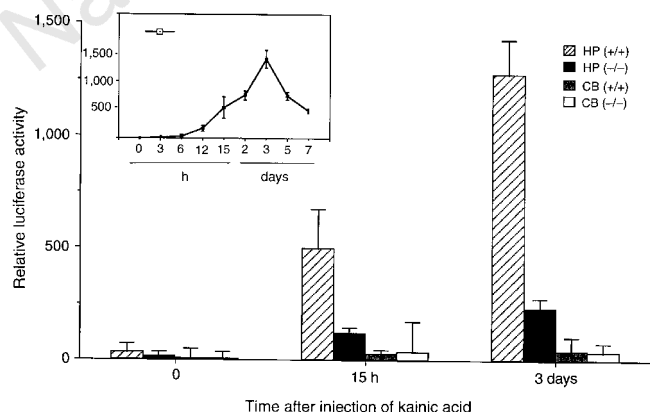


Figure 4 Kainic acid-induced AP-1 transcriptional activity in wild-type (+/+) and *Jnk3* knockout ($-/-$) mice expressing the AP-1 luciferase reporter transgene. Mice were killed at the times indicated after injection of kainic acid (30 mg per kg, i.p.). The relative luciferase activity in the hippocampus (HP) and the cerebellum (CB) was obtained by subtracting the activity following saline injection from the activity after injection with kainic acid. Each time point represents the mean ($\pm$ s.e.m.) of 3 to 5 individual animals. Inset, induction of AP-1 transcriptional activity in the hippocampus of wild-type mice after administration of kainic acid.

It is intriguing that continuing cell destruction, as indicated by the TUNEL assay, was observed 5 days after a transient episode of kainic acid-induced seizures. This continuous cell destruction is consistent with the reported delayed neurotoxicity of excitatory amino acids *in vitro*²⁵. Excitatory amino acids are reported to cause both acute necrotic cell damage and a delayed neurotoxicity predominated by apoptotic features in cultured neurons²⁶. To explore further the nature of the kainic acid-induced delayed cell death, we used toluidine-stained semithin sections and electron microscopy to investigate the ultrastructural changes to the degenerated hippocampal neurons. Hippocampal neurons in the *Jnk3*($-/-$) mice following kainic-acid injection contained distinctive apical dendrites and homogeneous euchromatin in the nuclei (Fig. 5j). In contrast, a large proportion of pyramidal neurons showing shattered apical dendrites and pyknotic nuclei were found in the CA1 hippocampal subfield of wild-type littermates (Fig. 5k). Electron microscopy revealed that the chromatin in the wild-type hippocampal nuclei was compacted and abutted on the inner surface of the nuclear envelope (asterisk in Fig. 5l), and there was also convolution of the nuclear outline (arrow in Fig. 5l) and condensation of the cytoplasm. Clusters of membrane-bound apoptotic bodies were also found in the damaged wild-type hippocampus (data not shown). Together, these morphological features are all consistent with the characteristics of apoptosis, and suggest a sustained alteration of intracellular homeostasis underlying the neurotoxicity of the systemic administration of kainic acid.

In conclusion, we tested the hypothesis that the selective expression of *Jnk3* in the nervous system may render neurons particularly vulnerable to environmental stress. In support of this hypothesis, we found that *Jnk3*-deficient mice exhibited a remarkable resistance to kainic acid-induced seizures and apoptosis. Importantly, the resistance to kainic-acid neurotoxicity in *Jnk3*($-/-$) mice was associated with decreased phosphorylation of c-Jun and AP-1 transcriptional activity under the same level of noxious stress, as indicated by the induction of c-Jun and c-Fos. These results indicate that the observed neuronal protection is due to the extinction of a *Jnk3*-mediated signalling pathway. Furthermore, we have recently generated *Jnk1*($-/-$) and *Jnk2*($-/-$) mice; these mice are readily

susceptible to seizures and apoptosis induced by a low dose of kainic acid (D. D. Y. *et al.*, unpublished data). Therefore, JNK isoforms may mediate differential responses to environmental stress with the *Jnk3* signalling pathway being particularly important in the pathogenesis of glutamate excitotoxicity. Because the amino-acid sequence of mouse, rat and human *Jnk3* is highly conserved^{6-8,27}, and the expression of the human *Jnk3* gene is also restricted to the nervous system^{7,27}, it is likely that the human and rodent *Jnk3* protein kinases may have very similar physiological functions. Neurotoxicity of the excitatory amino acids has been implicated in many neurological disorders, ranging from acute ischaemia to chronic neurodegenerative diseases^{1-28,29}. If the *Jnk3*-mediated apoptotic response we have identified is also involved in other types of excitatory neurotoxicity, *Jnk3* represents a promising subcellular target for future therapeutic intervention. □

Methods

Targeted disruption of the *Jnk3* gene. A 10-kb *NotI*-*EcoRI* (the *NotI* site was vector-derived) *Jnk3* fragment was cloned from a λ FixII phage library of a 129/Sv mouse strain (Stratagene). The targeting vector was constructed by inserting a 4.0-kb *MscI* fragment from the 5' end of the *Jnk3* genomic fragment, a 1.6-kb PGK-neo cassette, and a 1.8-kb *SpeI*-*NcoI* fragment of the 3' end of the *Jnk3* fragment into pBluescript KS vector (Stratagene) using appropriate linkers. The targeting vector contains a 2.6-kb PGK tk cassette flanking the 5' end of the *Jnk3* genomic sequence for negative selection of mutant ES cells. The resulting construct was linearized with *NotI* and introduced into W9.5 ES cells. Genomic DNA from transfectants resistant to G418 ($200 \mu\text{g ml}^{-1}$) (Gibco BRL) and gancyclovir ($2 \mu\text{M}$) (Syntex, Palo Alto, CA) were isolated and screened by Southern blot analysis.

Hybridization and protein kinase assays. Genomic DNA isolated from mouse tails was digested with *EcoRI* and examined by Southern blot analysis. The probe was prepared from the 5' region of the *Jnk3* genomic sequence (Fig. 1a). This probe hybridizes to a 12-kb fragment (endogenous *Jnk3* gene) and to a 4.2-kb fragment (disrupted allele). Northern blot analysis was performed using total RNA ($10 \mu\text{g}$) prepared from mouse tissues using the TRIzol Reagent (Gibco BRL). The blots were hybridized to a radiolabelled probe corresponding to nucleotides 62–412 of the murine *Jnk3* cDNA and a 421-base pair (bp) fragment corresponding to nucleotides 625–1,045 of the

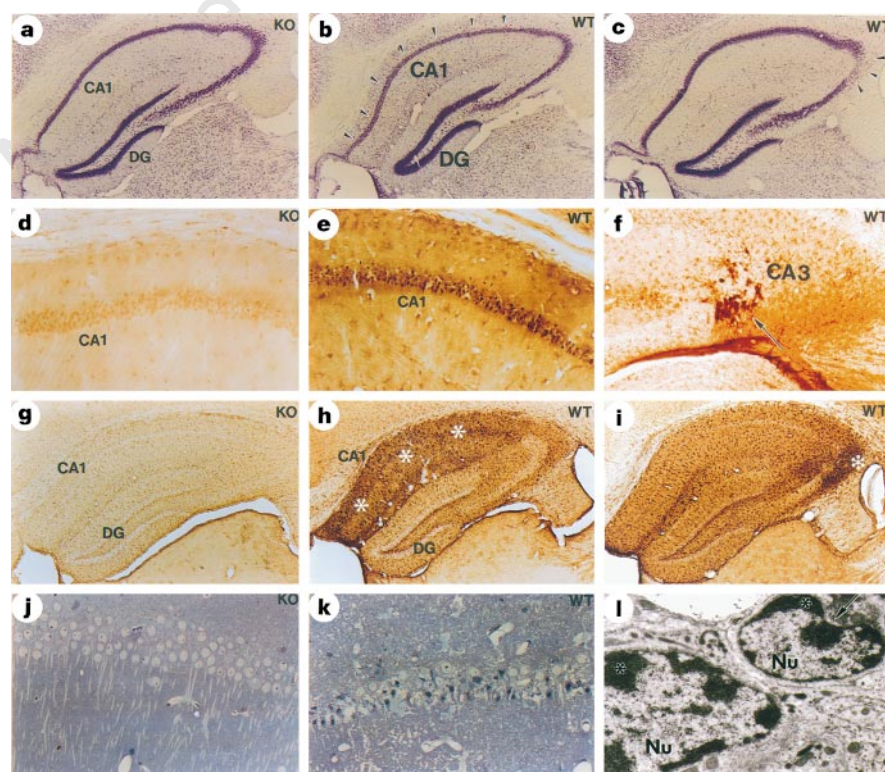


Figure 5 Comparison of kainic acid-induced cell damage in the hippocampus in wild-type and *Jnk3* knockout mice. The first row of sections (a–c) are stained by Nissl's method, the second row (d–f) are stained by TUNEL assay, the third row (g–i) are immunostained with GFAP antibody, and the fourth row (j–l) are 1- μm semithin sections stained by toluidine blue (j, k) and an electron micrograph of nuclear damage (l). The histological profile of *Jnk3*($-/-$) mice (the first column, a, d, g, j) is indistinguishable from unchallenged wild-type mice. Two patterns of kainic acid-induced cell death were found in wild-type mice: cell damage involving the hippocampal CA1 region (second column, b, e, h, k), and cell damage restricted to the hippocampal CA3 region (third column, c, f, i). Note the consistency of the damaged area (indicated by arrows and asterisks) demarcated by Nissl's staining (b, c), TUNEL labelling (e, f), and increased staining of GFAP (h, i). l, Compaction and marginalization of the chromatin (asterisk) in the nucleus (Nu) or pyramidal neurons was found in wild-type mice. There was also convolution of the nuclear envelope (arrow) and condensation of the cytoplasm. KO, knockout; WT, wild type. Original magnification: a–c, g–i, $\times 50$; f, $\times 100$; d, e, $\times 200$; j, k, $\times 400$; l, $\times 3,000$.

murine Jnk1 cDNA. To test *c-jun* and *c-fos* expression, a 199-bp fragment corresponding to nucleotides 891–1,089 of the murine *c-jun* cDNA and a 346-bp fragment corresponding to nucleotides 2,173–2,518 of the murine *c-fos* cDNA were used for the generation of radiolabelled probes for northern hybridization analysis. JNK activity in hippocampal lysates (30 µg) was measured before and after immunodepletion of Jnk1 and Jnk2 by in-gel protein kinase assays using the substrate glutathione S-transferase (GST)–cJun⁹.

Luciferase activity assay. Mice were decapitated and the brains dissected. Brain tissues were immediately lysed (Promega) and the luciferase activity was measured as described²⁴.

Kainic acid-induced expression of immediate-early genes. Homozygous mutant and control wild-type mice were killed and fixed by transcardial perfusion of 4% paraformaldehyde 2 or 6 h after the injection of kainic acid (30 mg per kg, i.p.). Brains from both groups were removed, post-fixed for 1 h, and sectioned on a vibratome (40 µm thick). Tissue sections were processed by immunocytochemistry to detect the expression of c-Jun (Santa Cruz), c-Fos (Santa Cruz), and phosphospecific c-Jun (Ser73) (New England Biolabs). Sections were floated in a solution of the primary antibody (diluted 200×) and incubated overnight at room temperature. Secondary antibody incubation, avidin–biotin conjugated peroxidase (Vectastain Elite ABC kit, Vector) and 3,3'-diaminobenzidine (Sigma) reactions were performed using standard procedures.

Kainic acid-induced hippocampal damage. Wild-type and *Jnk3*(–/–) mice were killed and fixed by transcardial perfusion of 4% paraformaldehyde and 1.5% glutaraldehyde 5 days after the injection of kainic acid (30 or 45 mg per kg, i.p.). Semithin and thin sections of brain were prepared using a vibratome and embedded in Epon. Tissue blocks were prepared using a microtome with a diamond tube for 1 µm-thick semithin sections examined by toluidine blue staining and for ultrathin sections examined by electron microscopy. Nissl's stain, GFAP immunocytochemistry, and TUNEL assays were performed using cryostat sections (50 µm) of cerebral hemispheres that were cryoprotected with sucrose. The TUNEL assay was modified from the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling assay. Briefly, tissue sections, directly mounted on a salinated slide, were permeabilized with 2% Triton X-100 (20 min at room temperature) and then incubated for nick end-labelling for 2 h at 37 °C using 0.32 U µl^{–1} TdT (Boehringer Mannheim) and 2 µM digoxigenin-11-dUTP (Boehringer Mannheim) in a final volume of 40 µl. The tissues were incubated with anti-digoxigenin antibody (Boehringer Mannheim) at 500× dilution and processed for immunocytochemistry using standard procedures.

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Identification and characterization of the vesicular GABA transporter

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Synaptic transmission involves the regulated exocytosis of vesicles filled with neurotransmitter. Classical transmitters are synthesized in the cytoplasm, and so must be transported into synaptic vesicles. Although the vesicular transporters for monoamines and acetylcholine have been identified, the proteins responsible for packaging the primary inhibitory and excitatory transmitters, γ-aminobutyric acid (GABA) and glutamate remain unknown^{1,2}. Studies in the nematode *Caenorhabditis elegans* have implicated the gene *unc-47* in the release of GABA³. Here we show that the sequence of *unc-47* predicts a protein with ten transmembrane domains, that the gene is expressed by GABA neurons, and that the protein colocalizes with synaptic vesicles. Further, a rat homologue of *unc-47* is expressed by central GABA neurons and confers vesicular GABA transport in transfected cells with kinetics and substrate specificity similar to those previously reported for synaptic vesicles from the brain. Comparison of this vesicular GABA transporter (VGAT) with a vesicular transporter for monoamines shows that there are differences in the bioenergetic dependence of transport, and these presumably account for the differences in structure. Thus VGAT is the first of a new family of neurotransmitter transporters.

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behaviours. Five genes have been identified that, when mutated, cause defects in these behaviours³. However, the phenotype of only one of these mutants, *unc-47*, is consistent with a loss of GABA transport into synaptic vesicles. First, the defect in *unc-47* is global, affecting all of the behaviours mediated by GABA. Second, the defect in *unc-47* is presynaptic, as the muscle cells in the mutant respond normally to GABA receptor agonists³. Third, GABA accumulates in GABAergic neurons of the mutant, suggesting that the neurotransmitter is not being released, possibly because it is not loaded into synaptic vesicles.

The *unc-47* gene maps between stP127 and *unc-50* on chromosome III of *C. elegans*. This region contains approximately 250 kilobases (kb) of DNA. Cosmids spanning this region were injected and two cosmid clones, T20G5 and E03F9, each rescued the *unc-47* mutant phenotype. The rescuing activity was further localized to a 5.2-kb *Bam*HI genomic fragment that contains a single complete open reading frame (ORF; T20G5.6) predicted by the *C. elegans* Genome Project⁸. To confirm that the identified ORF corresponds to the gene mutated in *unc-47* worms, three ethyl methanesulphonate-induced alleles were sequenced. The reference allele, *e307* (ref. 9), is a G to A transition of the absolutely conserved G in the splice acceptor site between exons five and six (Fig. 1). A second strong mutation, *n2476*, is a 238-base-pair deletion that removes parts of exons three and four; this deletion causes a frameshift at residue 175 and the ORF terminates after another 115 amino acids, indicating that this allele is a molecular null. Finally, *n2409* is a G to A transition which changes a glycine to arginine at residue 462 in a predicted transmembrane segment.

The cDNA derived from the *unc-47* gene predicts a protein of 486 amino acids (Fig. 1a) with no similarity to the previously characterized vesicular monoamine and ACh transporters^{4,6}. However, the sequence predicts ten transmembrane domains (TMDs) consistent with its being involved in transport (Fig. 1b). Further, a search of available databases with the UNC-47 protein sequence revealed that UNC-47 has weak sequence similarity to at least four predicted proteins in *C. elegans*, seven predicted proteins in the yeast *Saccharomyces cerevisiae*, and previously characterized amino-acid permeases in the plants *Arabidopsis thaliana* and *Nicotiana sylvestris* (Fig. 1d), suggesting that UNC-47 belongs to a class of proteins involved in the transport of amino acids.

To identify the cells that express *unc-47*, the protein coding sequence of green fluorescent protein (GFP)¹⁰ was inserted in-frame 52 amino acids downstream of the UNC-47 translation start site and co-injected with a *lin-15*⁺ (S. G. Clark & X. Lu,

personal communication)¹¹ marker gene into *lin-15(n765ts)* mutant animals¹². Animals containing the *unc-47::GFP* reporter construct showed expression of GFP in all GABAergic neurons, and only in GABAergic neurons (Fig. 2a), further supporting the idea that *unc-47* is involved in GABA transport.

To determine whether UNC-47 associates with synaptic vesicles, GFP was inserted at the carboxy terminus of the UNC-47 protein. This construct rescued the *unc-47* mutant phenotype, demonstrating that the construct functions normally. GFP-tagged UNC-47 was localized to synaptic varicosities along the ventral and dorsal cords but not in axons (Fig. 2b), a distribution similar to that of other synaptic vesicle proteins such as synaptobrevin¹³, synaptotagmin¹⁴, and Rab3a¹⁵. Further, mislocalization of synaptic vesicles in the *unc-104* mutant (which lacks a neuron-specific kinesin¹⁶) also results in mislocalization of UNC-47 (Fig. 2c). In *unc-104* mutants, synaptic vesicles do not reach the neuromuscular junction and accumulate in motor-neuron cell bodies¹³. Similarly, in *unc-104* mutants, GFP-tagged UNC-47 is present only in cell bodies and is not transported to the neuromuscular junction (Fig. 2c). Thus the sequence, distribution and subcellular localization of UNC-47 is consistent with its being involved in the packaging of GABA into synaptic vesicles.

To assess the biochemical function of UNC-47, we isolated the cDNA of a vertebrate homologue. A database search with the predicted peptide sequence of *unc-47* identified multiple entries in the mouse expressed sequence tag (EST) database. A fragment of one EST was amplified by PCR and used to screen a mouse brain cDNA library. Partial sequence of a 2.5-kb cDNA showed strong similarity to *unc-47*, and this cDNA was in turn used to screen a rat brain cDNA library. The resulting rat cDNA sequence contains a 3' untranslated region with ~95% identity to mouse and human but not *C. elegans* sequences (data not shown), a level exceeding that observed in much of the translated domain. The sequence of the largest ORF predicts a protein of 525 amino acids with 38% identity and 56% similarity to *unc-47* (Fig. 1a). Similar to UNC-47, the analysis of hydrophobic moment suggests that there are ten TMDs, and the absence of a single peptide predicts that the amino and the carboxy termini reside in the cytoplasm (Fig. 1b). In addition, the hydrophilic N-terminal domain is unusually large (~132 residues). The hydrophilic loops predicted to reside in the lumen of the vesicle lack consensus sites for N-linked glycosylation. However, consensus sequences for phosphorylation by protein kinase C occur on predicted cytoplasmic domains near the N terminus (+17), between TMDs 2 and 3 (+239) and between TMDs 6 and 7 (+377).

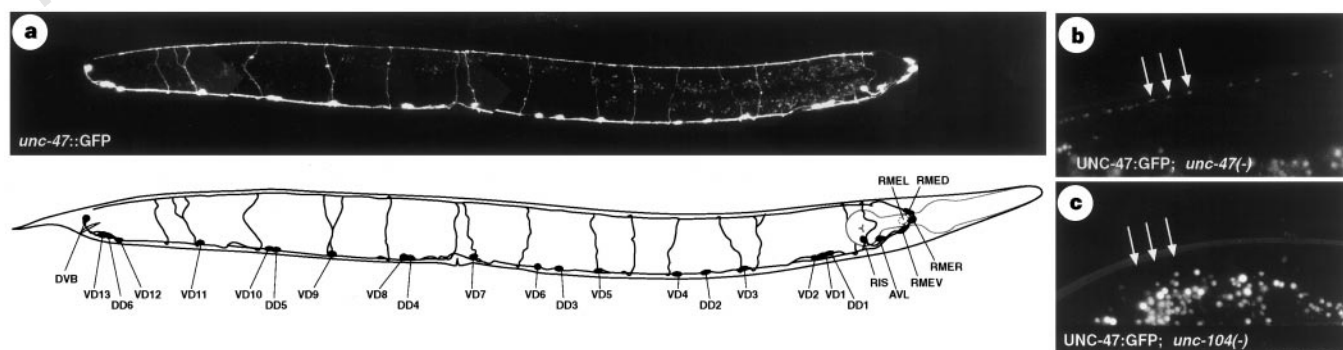


Figure 2 The *unc-47* gene is transcribed in GABAergic neurons, and the protein colocalizes with synaptic vesicles. **a**, Distribution of neurons that express *unc-47*. A confocal image and camera lucida drawing are shown of an adult *oxIn12* worm, which contains an integrated array of an *unc-47::GFP* reporter construct. GFP is expressed only in the cell bodies and axons of the 26 GABAergic neurons. **b**, Distribution of GFP-tagged UNC-47 protein. An *unc-47(e307)* mutant worm that contains an extrachromosomal array (*oxEx68*) of an UNC-47 GFP translational fusion construct. Confocal image of the dorsal cord at the posterior reflex of the

gonad. GFP fluorescence accumulates in synaptic varicosities of the DD motor neurons (arrows). The same distribution of UNC-47::GFP is observed in wild-type animals (not shown). **c**, Colocalization of UNC-47::GFP with synaptic vesicles in *unc-104(e1265)* mutant, which contains an UNC-47::GFP translational fusion construct. GFP is not observed at the nerve terminals of *unc-104* mutants but is only found in the cell bodies. Round spots are autofluorescent gut granules and are not associated with GFP expression.

To determine whether the rat *unc-47* homologue may function presynaptically in GABAergic transmission, we examined its tissue distribution. Northern analysis shows expression of an ~2.5 kb mRNA transcript in the brain, with none detected in non-neural tissues (Fig. 3). However, PCR amplification of reverse-transcribed sequences from spleen, testis and pancreas, but not liver or kidney, indicate expression of the *unc-47* homologue (data not shown), consistent with the detection of GABA biosynthesis and transport in some of these tissues¹⁷. *In situ* hybridization further demonstrates expression of the *unc-47* homologue throughout the neuraxis, but at particularly high levels within the neocortex, hippocampus, cerebellum, striatum, septal nuclei and the reticular nucleus of the thalamus (Fig. 4a–l), regions containing abundant GABAergic neurons. Examination of the autoradiograms under high magnification indicates expression by Purkinje cells of the cerebellum, as well as by interneurons of the cerebellum, hippocampus (Fig. 4m, n) and cortex, which are cell populations known to release GABA. We also performed *in situ* hybridization with one isoform of the biosynthetic enzyme glutamic acid decarboxylase (GAD), GAD-67 (Fig. 4). Although the level of hybridization in different regions varied slightly between the *unc-47* homologue and GAD-67, we found striking colocalization of the two sequences, consistent with the homologue functioning in the release of GABA. We also stained primary hippocampal cultures with an antibody that we raised to the *unc-47* homologue (R.J.R., S.L.M., R.H.E., manuscript in preparation). Punctate immunoreactivity in nerve processes that coincides with the immunoreactivity for synaptophysin is shown in Fig. 4o, p. The apparent vesicular localization further supports the *unc-47* homologue's being involved in vesicular GABA transport.

To determine biochemically whether the rat *unc-47* homologue encodes the vesicular GABA transporter, we expressed the cDNA in rat pheochromocytoma PC12 cells. PC12 cells contain synaptic-like microvesicles and support the activities of the vesicular monoamine transporters¹⁸ as well as the ACh transporter¹⁹, but do not express detectable amounts of *unc-47* homologue (data not shown). Stable PC12 transformants expressing high levels of the putative transport protein were isolated by screening cell clones with antibodies generated against the *unc-47* homologue. Immunofluorescence reveals a punctate pattern that is consistent with localization to intracellular membrane vesicles (data not shown). A population of light vesicles was then isolated from two stable transformants and purified by differential centrifugation. Membranes prepared in this way from the transfected cells show accumulation of ³H-GABA that is considerably greater than the background uptake observed in membranes from untransfected cells (Fig. 5a). The transport activity present in transfected PC12 cells saturates with increasing concentrations of GABA and has a *K_m* of ~5 mM (Fig. 5b), consistent with previous observations using rat brain synaptic vesicles^{20–23}. Thus the *unc-47* homologue is a vesicular GABA transporter (VGAT).

There are no known potent and specific inhibitors of vesicular GABA transport, so we have characterized in detail the functional

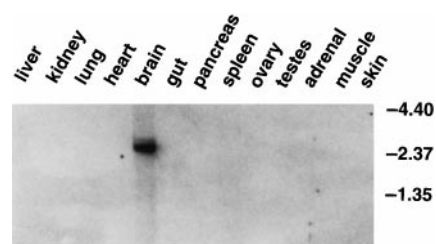


Figure 3 Brain-specific expression of the rat *unc-47* homologue. Northern analysis of poly(A)⁺ RNA prepared from different tissues shows a 2.5-kb mRNA transcript hybridizing with the rat *unc-47* cDNA only in the brain. The size of standards (kb) are shown on the right.

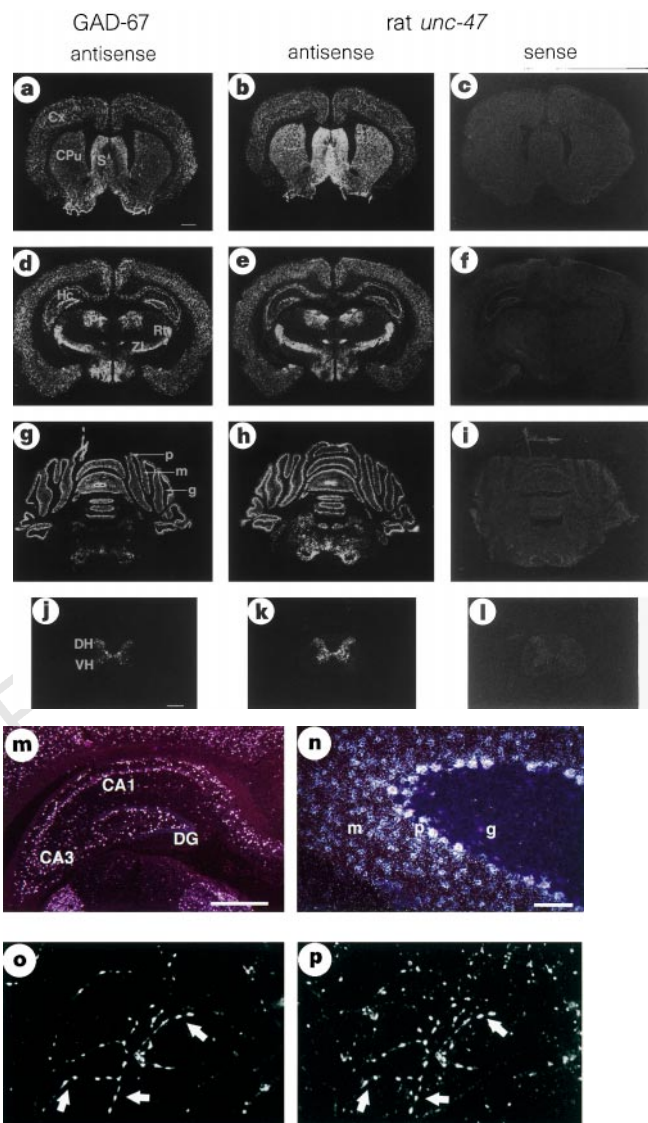


Figure 4 *In situ* hybridization shows expression of the rat *unc-47* homologue in GABAergic cell populations. **a–l**, ³⁵S-labelled antisense RNA probes for GAD-67 (**a, d, g, j**) and the rat *unc-47* homologue (**b, e, h, k**) and a sense probe for the homologue (**c, f, i, l**) were hybridized with sections through the basal ganglia (**a–c**), thalamus (**d–f**), cerebellum (**g–i**) and spinal cord (**j–l**). The pattern of hybridization by the *unc-47* homologue antisense probe appears identical to the antisense probe for GAD67, with no significant hybridization by the sense probes for the *unc-47* homologue or GAD-67 (not shown). Cx, cortex; CPu, caudate-putamen; S, septal nuclei; Hc, hippocampus; Pt, prefrontal nuclei; Rt, reticular nucleus of the thalamus; Zl, zona incerta; Hy, hypothalamus; p, Purkinje cell layer of the cerebellum; m, molecular layer; g, granule cell layer; DH, dorsal horn of the spinal cord; and VH, ventral horn. Scale bars indicate 1 mm, and the bar in **a** applies to **a–i**, the bar in **j** to **j–l**. **m, n**, Brain sections hybridized with ³⁵S-labelled antisense RNA for the *unc-47* homologue were counter-stained with cresyl violet and viewed under darkfield illumination. **m**, The hippocampus shows expression in the hilus of the dentate gyrus (DG) and regions CA1 and CA3. Minimal staining occurs in the granule cells of the DG and in pyramidal cells. **n**, The cerebellum shows strong hybridization in the Purkinje cell (p) and molecular layers, with little hybridization in the granule cell layer. Hybridization with the GAD-67 probe shows a similar pattern and sense probes show no signal (data not shown). Scale bars indicate 1 mm (**m**) and 0.1 mm (**n**). **o, p**, Double immunofluorescence of primary hippocampal cultures for the *unc-47* homologue (**o**) coincides with immunoreactivity for synaptophysin (**p**). The cells were examined under epifluorescence at ×63 magnification. The arrows indicate processes with coincident staining for both the *unc-47* homologue and synaptophysin. The synaptophysin immunoreactivity that does not correspond to staining for the *unc-47* homologue presumably reflects the presence of non-GABAergic neurons in the culture.

properties encoded by the rat VGAT (rVGAT) cDNA to determine its relationship to the activity previously described in native brain synaptic vesicles. To assess ligand recognition, we first considered compounds structurally related to GABA that have been tested as inhibitors of GABA transport into synaptic vesicles. As for GABA transport into synaptic vesicles, the plasma membrane GABA transport inhibitor nipecotic acid inhibits rVGAT activity only weakly (Fig. 5c), and the excitatory amino-acid transmitter glutamate does not inhibit rVGAT activity even at extremely high concentrations (Table 1)^{24–26}. Thus the analysis of ligand specificity also distinguishes rVGAT from these other neurotransmitter transporters. The GABA analogue *N*-butyric acid weakly inhibits both rVGAT activity and GABA transport into brain synaptic vesicles, further supporting the identity of rVGAT as a vesicular GABA transporter. Previous studies using synaptic vesicles have also suggested the inhibition of vesicular GABA transport by γ -vinyl-GABA (vigabatrin), an inhibitor of GABA transaminase and a potent anticonvulsant²⁷. This synthetic GABA analogue also inhibits the transport of ³H-GABA by rVGAT as potently as unlabelled GABA, supporting the idea that there is an additional site of action for this drug.

Previous studies of native brain synaptic vesicles suggested that a single vesicular GABA transport activity also recognizes the inhibitory amino-acid transmitter glycine as a substrate²³. However, other studies suggest that glycine has a different transporter²². Glycine

inhibits GABA transport encoded by the rVGAT cDNA with relatively low potency. We failed to detect significant uptake of ³H-glycine in these preparations (data not shown), supporting the existence of a distinct vesicular transporter for glycine.

Functional analysis of rVGAT indicates bioenergetic differences from vesicular transporters for monoamines and ACh. Monoamine and ACh transport rely primarily on the chemical component ($\Delta\mu\text{H}^+$) of the proton electrochemical gradient ($\Delta\mu_{\text{H}^+}$)^{1,2}. Studies using synaptic vesicles have indicated that vesicular GABA transport relies on the electrical component $\Delta\Psi$, as well as ΔpH ^{21–23}. To assess the bioenergetics of rVGAT function, we used the ionophore nigericin, which exchanges K^+ for H^+ , to selectively dissipate ΔpH . Nigericin reduces rVGAT activity by ~40%, suggesting that $\Delta\Psi$ has an additional role (Fig. 5d). Indeed, the addition of both nigericin and valinomycin, an ionophore that mediates K^+ flux and so dissipates $\Delta\Psi$ as well as ΔpH , eliminates rVGAT activity. To compare directly the bioenergetics of vesicular GABA and monoamine transport, we have examined the transport of serotonin by the same PC12 cell-membrane preparations that contain rVGAT. Previous work has demonstrated the expression of endogenous vesicular monoamine transporter 1 (VMAT1) on synaptic-like microvesicles in these cells¹⁸. Nigericin inhibits VMAT1 activity on these vesicles to a greater extent (~65%) than it inhibits rVGAT activity, indicating a greater dependence on ΔpH (Fig. 5d). The addition of valinomycin to nigericin eliminates the residual VMAT1

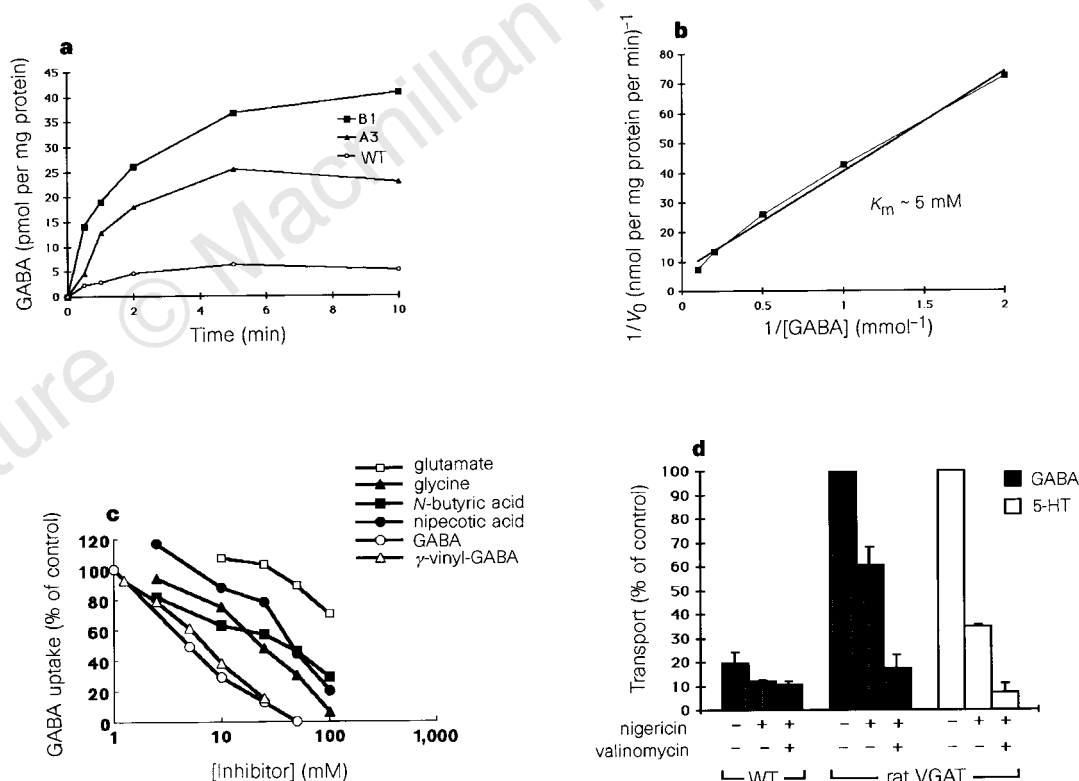


Figure 5 The rat *unc-47* homologue encodes vesicular GABA transport. **a**, Membranes prepared from two different PC12 cell clones (A3 and B1) stably expressing the rat *unc-47* homologue accumulate more ³H-GABA than membranes prepared from untransfected PC12 cells (WT). **b**, Lineweaver-Burke plot of initial, maximal transport rate (V_0) in the presence of different concentrations of GABA (μM), with the linear approximation performed by standard regression analysis. **c**, Inhibition of GABA transport activity by GABA and related compounds. γ -Vinyl-GABA inhibits almost as potently as GABA itself, whereas glycine inhibits much less potently and glutamate inhibits only very slightly (see Table 1 for IC_{50} values). *N*-butyric acid and nipecotic acid, an inhibitor of plasma membrane GABA transport, inhibit vesicular GABA transport very weakly. **d**, Vesicular GABA transport and vesicular monoamine transport differ in

bioenergetics. Membranes prepared from PC12 cell clone B1 stably expressing rVGAT (middle) show considerably more GABA transport activity (filled bars) than membranes from untransfected cells (WT, left). Nigericin ($5\mu\text{M}$) inhibits GABA transport in the transfected cells by ~40%, and the addition of valinomycin ($20\mu\text{M}$) to nigericin eliminates rVGAT activity, indicating greater dependence on $\Delta\Psi$ than ΔpH . In contrast, transport of serotonin by endogenous VMAT1 (white bars) expressed in the same membranes from transfected PC12 cells that express rVGAT (right) shows ~65% inhibition by nigericin, indicating that VMAT1 depends more on ΔpH than rVGAT. The results are normalized to GABA transport (left and middle) and serotonin transport (right) by rVGAT-expressing cells. Error bars represent the standard error of the mean.

Table 1 Inhibition of GABA transport by structurally related compounds

Inhibitor	IC ₅₀ (mM)	s.e.
GABA	4.75	0.3
γ-Vinyl-GABA	7.5	0.7
Glycine	27.5	10.6
N-butyric acid	43.5	2.1
Nipecotic acid	46	1.4
Glutamate	>100	

Membranes expressing rVGAT were incubated in 2 μCi ³H-GABA for 5 min at 29°C. The concentrations of structurally related compounds required to inhibit the accumulation of ³H-GABA by 50% (IC₅₀) are indicated, along with the standard errors (s.e.).

activity, indicating a small role for ΔΨ. Thus rVGAT and VMAT1 depend on both components of the electrochemical gradient, but VMAT1 depends to a greater extent on ΔpH, which is consistent with previous results using mixed populations of synaptic vesicles.

Identification of the *unc-47* homologue as a vesicular GABA transporter helps us to understand the molecular mechanism for the vesicular transport of an amino-acid neurotransmitter. The genetic analysis in *C. elegans* indicates that *unc-47* is essential for GABA transmission. In addition, UNC-47 and its rat homologue both occur in GABAergic neurons, and their polytopic nature supports the idea that they function in vesicular transport. Biochemical characterization of the rat *unc-47* homologue demonstrates GABA transport function with the affinity and ligand specificity reported for GABA transport by native synaptic vesicles. We cannot detect transport by rVGAT of the other principal inhibitory transmitter glycine, suggesting the existence of a distinct vesicular glycine transporter. The analysis of GABA and monoamine transport activities expressed in the same population of membrane vesicles shows that rVGAT is more dependent on ΔΨ than is VMAT1. Indeed, this difference in bioenergetic mechanism may account for the structural differences between these two classes of vesicular transporters. UNC-47 and rVGAT show similarity to a large group of sequences from *C. elegans*, *S. cerevisiae* and plants. Several of the plant sequences mediate amino-acid transport and use a proton electrochemical gradient as the driving force²⁸, suggesting functional as well as structural similarity to UNC-47 and rVGAT. However, these plant permeases catalyse the co-transport of amino acids and protons rather than the proton exchange mediated by rVGAT, suggesting that the sequence similarity reflects general features of substrate recognition and proton movement, rather than the precise mechanism of bioenergetic coupling. The class of proteins identified by UNC-47 and rVGAT may include the transporters for excitatory amino-acid transmitters, such as glutamate, which also depend on ΔΨ. □

Methods

Cloning of *unc-47*. A pool containing 10 ng μl⁻¹ each of cosmid E03F9, ZK1128, F55E6, T20G5 and K08E5 was injected along with 80 ng μl⁻¹ of EK L15 (*lin-15*⁺) marker plasmid (S. G. Clark & X. Lu, personal communication)¹¹. The DNA was injected into the syncytial gonads of *unc-47*(e307); *lin-15* (*n765ts*) animals. Five *lin-15*⁺ lines were established and all animals were rescued for the *unc-47* mutant phenotypes. A 5.2-kb *Bam*HI fragment subcloned from T20G5 rescued *unc-47*(e307) mutants. The 5.2-kb *Bam*HI fragment was used to screen 350,000 plaques from an oligo-dT-primed λZAP cDNA library made from mixed-stage RNA and a single positive (B1) was identified. To isolate additional cDNAs, 400,000 plaques of a second oligo-dT-primed mixed-stage cDNA library were screened and four positives were isolated (OK1-4). Sequence analysis showed that the B1 cDNA uses an alternative splice donor site in exon five, resulting in deletion of the eighth and part of the ninth TMDs. However, PCR amplification of reverse-transcribed cDNA demonstrated that this mRNA transcript is extremely rare and presumably results from aberrant splicing. The 5' end of *unc-47* was identified by PCR amplification of first-strand cDNA prepared from mixed-stage poly(A)⁺ RNA. Sequence analysis of the product revealed an SL1 splice leader immediately 5' to the ATG start codon. To prepare genomic DNA from mutant alleles e307, n2476, and n2409, approximately 20 homozygous mutant worms were washed three times with M9 salts to remove

bacteria, resuspended in 5 μl water, and boiled for 5 min. TE (4 μl), 8.0, and 1 mg ml⁻¹ proteinase K (1 μl) were added and the mixture incubated at 45°C for 1 h. Proteinase K was inactivated by boiling for 30 min, and 1 μl of this DNA preparation was used for PCR amplification. Amplified fragments were purified and sequenced using the Cyclist *Taq* DNA sequencing kit (Stratagene).

GFP expression constructs. To construct the U47GFPNTX transcriptional fusion, GFP and *unc-54* RNA termination sequences were amplified from pPD95.85 (S. Xu & A. Fire, personal communication) using primers that engineered an in-frame *Bsp*HI site onto the 5' end of GFP. Using an internal *Bsp*HI site, which includes 553 bp of the *unc-54* terminator, the amplified fragment was cloned into the *Bsp*HI site in the *unc-47*-rescuing 5.2-kb *Bam*HI fragment. U47GFPNTX (30 ng μl⁻¹) and 35 ng μl⁻¹ EK L15 (*lin-15*⁺) DNA were injected into the gonads of *lin-15*(n765ts) mutants. Three lines carrying an extrachromosomal array of both U47GFPNTX and EK L15 were established, and all three lines expressed GFP in the GABAergic neurons. The extrachromosomal array from one line was integrated into chromosome X by X-ray integration to generate the strain EG1285: *lin-15*(n765ts); *oxIn12*. To construct the U47GFPCTL translational fusion, a *Sal*I site was engineered into the C terminus of *unc-47* protein-coding sequence, two residues N-terminal to the UAA stop codon. GFP was amplified by PCR with primers that engineered an in-frame *Sal*I site onto each end of the GFP fragment and then cloned into the *Sal*I site created at the C terminus of UNC-47. U47GFPCTL (30 ng μl⁻¹) and 35 ng μl⁻¹ EK L15 (*lin-15*⁺) DNA were injected into the gonads of *lin-15*(n765ts) and into *lin-15*(n765ts); *unc-47*(e307) mutant worms, and three *lin-15*(n765ts) lines were established that contained an extrachromosomal array of both U47GFPCTL and *lin-15*⁺. Four *lin-15*(n765ts); *unc-47*(e307) lines were obtained that contained both U47GFPCTL and *lin-15*⁺ in an extrachromosomal array. Worms in two lines had strong GFP expression at the nerve terminals and almost all *lin-15*⁺ animals were also *unc-47*⁺. To express U47GFPCTL in the *unc-104*(e1265) mutant background, the *oxEx68* [U47GFPCTL; *lin-15*⁺] extrachromosomal array was crossed into *unc-104*(e1265); *lin-15*(n765ts) mutants to generate EG1300: *unc-104*; *lin-15*; *oxEx68*.

PCR amplification and library screening. A search of the available EST database with the predicted amino-acid sequence of UNC-47 identified mouse EST 252177 as a possible vertebrate homologue. Using oligonucleotide primers based on 252177, a fragment was amplified by PCR from a pooled mouse brain cDNA library. Briefly, 200 ng template DNA was amplified in a 50-μl reaction containing 25 mM Tris, pH 8.3, 50 mM KCl, 3 mM MgCl₂, 100 pmol of each oligonucleotide, 100 μM dNTPs, and 1 μl *Taq* polymerase for 30 cycles involving the denaturation at 92°C for 1 min, annealing at 66°C for 1 min, and extension at 72°C for 1 min. After gel purification, the fragment was radiolabelled by PCR amplification under similar conditions in the presence of 2 μM non-radioactive dCTP and 1 μM ³²P-dCTP and used to screen a mouse brain bacteriophage cDNA library by aqueous hybridization at 47.5°C (ref. 4). After washing at 52°C, positively hybridizing phage were identified by autoradiography, purified by two sequential rounds of screening, and the cDNA inserts rescued. After confirmation of the close sequence similarity to *unc-47*, this fragment was radiolabelled by random priming and used to screen a rat brain bacteriophage cDNA library as described above. After characterization of the resulting cDNA clones, the 5' end of the cDNA was amplified by PCR from a different rat brain cDNA library using oligonucleotide primers from the known rat sequence together with a primer flanking the vector insertion site. Another oligonucleotide primer based on the additional sequence was then used to amplify the 5' end of the cDNA from the library with *Pfu* polymerase rather than *Taq* polymerase to minimize mutations. This 5' fragment was then joined to a cDNA clone isolated by hybridization at a common *Bgl*II restriction site. The dideoxy chain termination method was used to confirm the sequence of the cDNA on both strands.

Northern analysis. Poly(A)⁺ RNA (5 μg) prepared from different rat tissues was separated by electrophoresis through formaldehyde-agarose and transferred to nylon membranes. Staining with ethidium bromide revealed approximately equal amounts of RNA in each lane. After hybridization in 50% formamide⁴ to the *unc-47* homologue cDNA radiolabelled by random priming, the filters were autoradiographed with an enhancing screen.

In situ hybridization. Adult rats were anaesthetized with pentobarbital and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). After dissection, the brains were postfixed in the same solution, cryoprotected in

30% sucrose/PBS, and 15- μ m sections hybridized at 52 °C in 50% formamide containing 0.3 M NaCl, 20 mM Tris, pH 7.4, 5 mM EDTA, 10 mM NaH₂PO₄, 1× Denhardt's solution, 10% dextran sulphate, and 0.5 mg ml⁻¹ yeast RNA to ³⁵S-labelled RNA probes transcribed from linearized plasmid templates and hydrolysed in alkali to ~300 nucleotide fragments²⁹. After washes in 50% formamide and digestion with RNase A, the slides were autoradiographed.

Immunofluorescence. Primary hippocampal cultures were grown on poly-D-lysine-coated glass coverslips for two weeks, fixed in 4% paraformaldehyde/PBS for 20 min, rinsed in PBS, blocked in 0.02% saponin, 2% BSA, 1% fish skin gelatin/PBS (blocking buffer) for 1 h and incubated for 90 min with anti-rVAT polyclonal rabbit and anti-synaptophysin monoclonal mouse antibodies diluted 1:100 in blocking buffer, all at room temperature. The cells were then washed, incubated in secondary anti-rabbit antibody conjugated to fluorescein and anti-mouse antibody conjugated to rhodamine (both Cappel) both diluted 1:100, washed, the coverslips mounted on class slides, and viewed under epifluorescence.

Membrane preparation. The rat *unc-47* homologue cDNA subcloned into the plasmid expression vector pcDNA3-Amp (Invitrogen) was introduced into PC12 cells by electroporation³⁰. The cells were then selected in 800 μ g ml⁻¹ G418 (effective) and the resulting clones examined by immunofluorescence¹⁸ using a rabbit polyclonal antibody (R.R., S.M. & R.H.E., manuscript in preparation). Using the two cell clones with the highest level of immunoreactivity, membranes were prepared by first resuspending the washed cells in 0.3 M sucrose, 10 mM HEPES-KOH, pH 7.4 (SH buffer) containing 0.2 mM diisopropylfluorophosphate (DFP), 1 μ g ml⁻¹ pepstatin, 2 μ g ml⁻¹ aprotinin, 2 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ E64 and 1.25 mM MgEGTA. The cells were then disrupted by homogenization at 4 °C through a ball-bearing device at a clearance of 10 μ m. The nuclear debris was sedimented at 1,000g for 5 min and heavier membranes were eliminated by centrifugation at 27,000g for 1 h. The remaining light membrane vesicles were sedimented at 65,000g for 1 h and resuspended in SH containing the same protease inhibitors at a final concentration of ~10 μ g protein per μ l.

Transport assay. To initiate the reaction, 10 μ l of membranes was added to 200 μ l SH buffer containing 4 mM MgCl₂, 4 mM KCl, 4 mM ATP, 40 μ M unlabelled GABA and 2 μ Ci ³H-GABA (NEN). Incubation was performed at 29 °C for varying intervals and the reaction was terminated by rapid filtration (Supor 200, Gelman), followed by immediate washing with 6 ml cold 0.15 M KCl. Background uptake was determined by incubation at 4 °C for 0 min. The bound radioactivity was measured by scintillation counting in 2.5 ml Cytosint (ICN). To determine K_m, unlabelled GABA was added at a range of concentrations and uptake were measured at 30 s. Nigericin and valinomycin dissolved in ethanol added to final concentrations of 5 μ M and 20 μ M, respectively. Transport measurements were performed in duplicate and repeated three or more times using at least two different membrane preparations.

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Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours

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Sporadic basal cell carcinoma (BCC) is the most common type of malignant cancer in fair-skinned adults. Familial BCCs and a fraction of sporadic BCCs have lost the function of Patched (Ptc), a Sonic hedgehog (Shh) receptor^{1–3} that acts negatively on this signalling pathway. Overexpression of Shh can induce BCCs in mice⁴. Here we show that ectopic expression of the zinc-finger transcription factor Gli1 in the embryonic frog epidermis results in the development of tumours that express endogenous Gli1. We also show that *Shh* and the *Gli* genes are normally expressed in hair follicles, and that human sporadic BCCs consistently express Gli1 but not *Shh* or Gli3. Because Gli1, but not Gli3, acts as a target and mediator of Shh signalling⁵, our results suggest that expression of Gli1 in basal cells induces BCC formation. Moreover, loss of Ptc or overexpression of Shh cannot be the sole causes of Gli1 induction and sporadic BCC formation, as they do not occur consistently. Thus any mutations leading to the expression of Gli1 in basal cells are predicted to induce BCC formation.

Gli1, which was originally isolated as an amplified gene in a glioma⁶, is a member of a multigene family^{7–9} and can transform

fibroblasts in cooperation with adenovirus E1A¹⁰. Ectopic expression of *Gli1* in frog embryos activates *Shh* target genes, including that encoding HNF-3 β , both in neural and epidermal non-neural ectoderm^{5,11}, showing that epidermal cells can respond to *Shh* signalling. Frog embryos injected with plasmids driving the expression of frog *Gli1* developed abnormal growths, or tumours, in the otherwise normal, smooth tadpole epidermis (78%, $n = 25$; Fig. 1a). The tumours were independent of HNF-3 β expression, as only a fraction contained cells expressing HNF-3 β (30%, $n = 27$). Because the plasmid DNA was targeted to the animal-most region of the two-cell embryo, only ectodermal derivatives inherit plasmids, indicating that the growths of focal epidermal hyperplasia (the tumours) observed are caused by expression of *Gli1* in the epidermis. Indeed, detection of epitope-tagged *Gli1* in injected embryos showed exclusive expression in the ectoderm⁵ (not shown). Histological sections of 1-week-old (stage ~45) injected tadpoles revealed tumours in the epidermis, sometimes consisting of densely packed cells (Fig. 1f). These cells were clearly distinct from all normal tissues (Fig. 1e) and did not express the cement-gland marker *XAG-1* (ref. 12) ($n = 10$; not shown). Taken together, these results show that transient epidermal expression of *Gli1* leads to tumour formation *in vivo*.

To determine whether tumours formed from cells inheriting injected *Gli1*, embryos were co-injected with frog *Gli1* RNA and *lacZ* RNA as tracer. Injected tadpoles showed prominent tumours of the skin (80%, $n = 12$) formed from the superposition of epidermal cells that inherited *Gli1*, as these invariably expressed β -galactosidase (Fig. 1b–d). Labelled epidermal cells located inside the tumours were distinct from the underlying lateral-plate mesoderm, which was always unlabelled. The uninjected side displayed typical smooth embryonic epidermis (Fig. 1c, right) and injection of *lacZ* alone had no effect. Because not all epidermal cells inheriting *Gli1* RNA become tumorigenic, there could be a requirement for a certain level of *Gli1* to initiate tumour formation. The effects of *Gli1* are specific, as injection of plasmids driving the expression of human *Gli3* (ref. 7) had no effect⁵ ($n = 45$; not shown). Inappropriate expression of *Gli1* therefore leads to tumour formation, although it is not clear if this represents epidermal neoplastic transformation.

The finding of mutated *Ptc* alleles in familial and some sporadic BCCs^{1–3} together with the development of skin tumours in tadpoles overexpressing *Gli1* raised the possibility that *Gli1* could be expressed in and underlie the development of sporadic adult basal cell cancer. Sections of freshly excised human BCCs were analysed by *in situ* hybridization. All but one of the samples examined showed unambiguous expression of *Gli1*, although the level of expression varied (46 of 47; Table 1, Fig. 2). The variability observed in *Gli1* RNA expression could be due to inherent differences in the tumours or to differences in the preservation of the excised material. No correlation was detected between the level of *Gli1* expression and the site or the aggressiveness of the tumour. In contrast to the consistent expression of *Gli1*, only 76% (23 of 30; Table 1) of the cases displayed unequivocal expression of *Gli3* (Fig. 2k), which is often coexpressed with *Gli1* (refs 5, 9, 13, 14). Analysis of ten cases of squamous cell carcinoma (SCC) *in situ* showed *Gli* gene expression to be absent (Table 1, Fig. 2s, t). Control hybridizations with sense RNA probes showed no signal (Table 1, Fig. 2l).

Tumour nodules infiltrating the dermis showed the highest levels of *Gli1* expression (Fig. 2a–f, i, j, n), and here it was often concentrated in the periphery (Fig. 2f, j), where most proliferating cells appear to be located¹⁵. In tumorigenic regions, the basal layer of the epidermis also displayed high levels of *Gli1* expression (Fig. 2c–e). The pattern of expression of *Gli3* was distinct from that of *Gli1* but was also detected primarily in the periphery of tumour nodules (Fig. 2k). *Gli* gene expression was not detected in the interfollicular epidermis or the dermis in normal regions distal from the tumour (Fig. 2q, r), although *Gli1* mRNA was detected in

histologically normal basal cells immediately surrounding the tumour site (Fig. 2a, b, right). Cells of the sebaceous glands, dermis and blood vessels were negative. Single cells surrounding the main BCC tumour masses were rarely positive for *Gli1* (3 of 47) or *Gli3* expression (1 of 30; Fig. 2j, k), and could represent early invading tumour cells that are histologically unrecognizable. Alternatively, these single cells may be non-BCC cells that express *Gli1* in response to a secreted tumour-derived factor.

Expression of *Gli1* was also analysed by immunocytochemistry with an affinity-purified anti-human *Gli1* polyclonal antibody⁵. All samples showed specific *Gli1* expression (6 of 6; Table 1; Fig. 2u–w). Control antibody labelling with an anti-HNF-3 β polyclonal antibody^{11,16} showed no specific labelling (Table 1 and data not shown). In BCCs, *Gli1* protein was detected in the cytoplasm (Fig. 2u, w), consistent with the prevalent cytoplasmic localization of frog *Gli1* (ref. 5). This is also consistent with an association of *Gli* proteins with the cytoskeleton¹⁷, but contrasts with the nuclear localization of *Gli1* in COS cells transfected with the glioma-derived cDNA⁵ (Fig. 2x) and in a glioma line showing a 75-fold overexpression of *Gli1* (D259MG^{6,18}). The glioma cDNA⁶ thus appears to encode a mutated protein that escapes cytoplasmic retention.

The possibility that *Gli1* could activate components of the *Shh* signal-transduction pathway in sporadic BCCs, including endogenous human *Shh* itself^{19,20}, was suggested by the regulatory loop

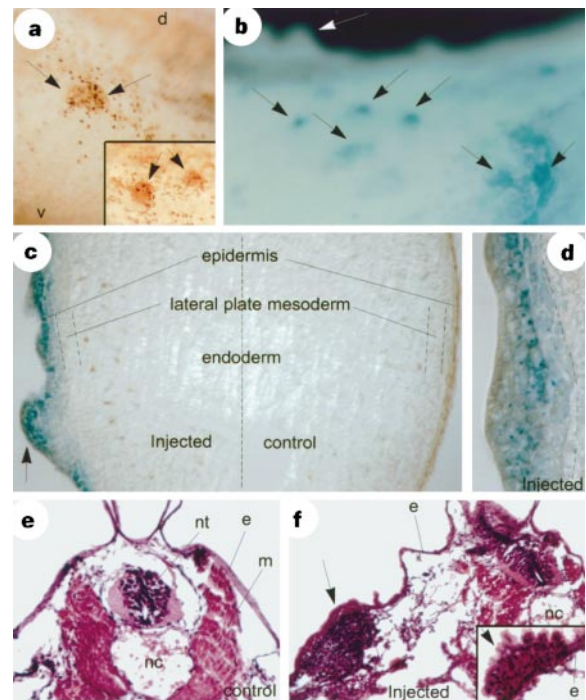


Figure 1 Ectopic expression of *Gli1* in frog embryos leads to the formation of epidermal tumours. **a, b**, Whole-mount view of the flank of injected tadpoles (stages ~32–34) showing epidermal tumours (arrows) and cells expressing HNF-3 β (brown). The embryo in **b** was co-injected with *Gli* synthetic RNA and *lacZ* RNA as tracer. β -Gal activity is shown in blue; d, dorsal; v, ventral. **c, d**, Histological cross-sections through the trunk of embryos unilaterally injected with *Gli1*. The affected side is blue (arrow, left in **c**). **d**, Detail of a tumour similar to that shown in **c** in which β -gal activity is detected as small cytoplasmic inclusions. The boundaries between the epidermis and the underlying lateral plate mesoderm and between this and the endoderm are denoted by broken lines. **e, f**, Histological sections stained with haematoxylin and eosin through the trunk of control (**e**) and *Gli1*-injected (**f**) stage ~45 tadpoles. An epidermal tumour is detected in the flank (arrow in **f**). The inset in **f** shows an outwardly growing epidermal tumour (arrow); e, epidermis; m, muscle; nc, notochord; nt, neural tube. Dorsal side is up in all cases. In **a** and **b**, anterior is to the left.

defined in the Shh signalling pathway in which Shh signalling triggers a cascade of events leading to the activation of *Gli1*, which in turn may activate the transcription of Shh target genes including *Shh* and *Ptc*. By *in situ* hybridization, 44% (15 of 34) of BCCs were positive for *Shh*, with expression localized to the tumour masses that also expressed *Gli1* (Fig. 2o, Table 1). Analysis of nine SCCs *in situ* showed no *Shh* expression (Table 1). Sense *Shh* RNA probes showed no specific signal (Table 1). Reverse transcription–polymerase chain reaction (RT–PCR) analyses revealed *Shh* expression in two cases of BCC, whereas this was below the level of detection in three BCCs and one SCC *in situ*. All these samples showed low levels of *Gli3* expression, and all five BCCs, but not the SCC, showed elevated levels of *Gli1* and *Ptc* mRNAs. Expression of the ribosomal gene *S17* was monitored as a control (not shown). The lower frequency of *Shh* (17 of 37 cases overall) compared with *Gli1* expression (49 of 50 cases overall) in BCCs, together with the inability of injected Shh to initiate epidermal tumour formation in frog embryos¹¹, suggests that Shh is unlikely to be the only cause of *Gli1* expression in BCCs, and that *Shh* may not be regulated solely by *Gli1*. In contrast, *Ptc* was expressed in all BCCs examined³ (16 of 16; Fig. 2p, Table 1 and not shown), and was coincident with *Gli1* and

Shh (Fig. 2m–o), consistent with *Ptc* being a target of *Gli1*.

Tadpole tumours induced by *Gli1* could be the equivalent of human BCCs. However, morphological analyses, the main criteria of dermatopathologists, cannot be applied to the tadpole tumours as early tadpoles do not have dermis. Our molecular analysis of BCCs provides an alternative test for the BCC-like nature of tadpole tumours, as endogenous *Gli1* is expressed consistently in the human tumours (Table 1, Fig. 2). Injection of human *Gli1*, which we have previously shown has a similar activity to frog *Gli1* (ref. 5), resulted in the formation of epidermal tumours (79%, $n = 24$), and all tumours displayed expression of the endogenous frog *Gli1* gene (Fig. 3a–c). Endogenous *Gli1* expression was not detected in non-tumorigenic regions of the epidermis in injected embryos or control siblings (Fig. 3a). In contrast, only a small fraction (12–23%) of embryos injected with *Gli1* mRNA or pDNA showed ectopic *Shh* expression, mostly in the non-tumorigenic epidermis of injected tadpoles (3 of 25 for RNA, Fig. 3e and 7 of 30 for pDNA) and early neurulae¹¹, not observed in controls (Fig. 3d). The low incidence of *Shh* expression and the consistent expression of *Gli1* in tadpole epidermal tumours parallels that found in BCCs and points to their having a BCC-like nature.

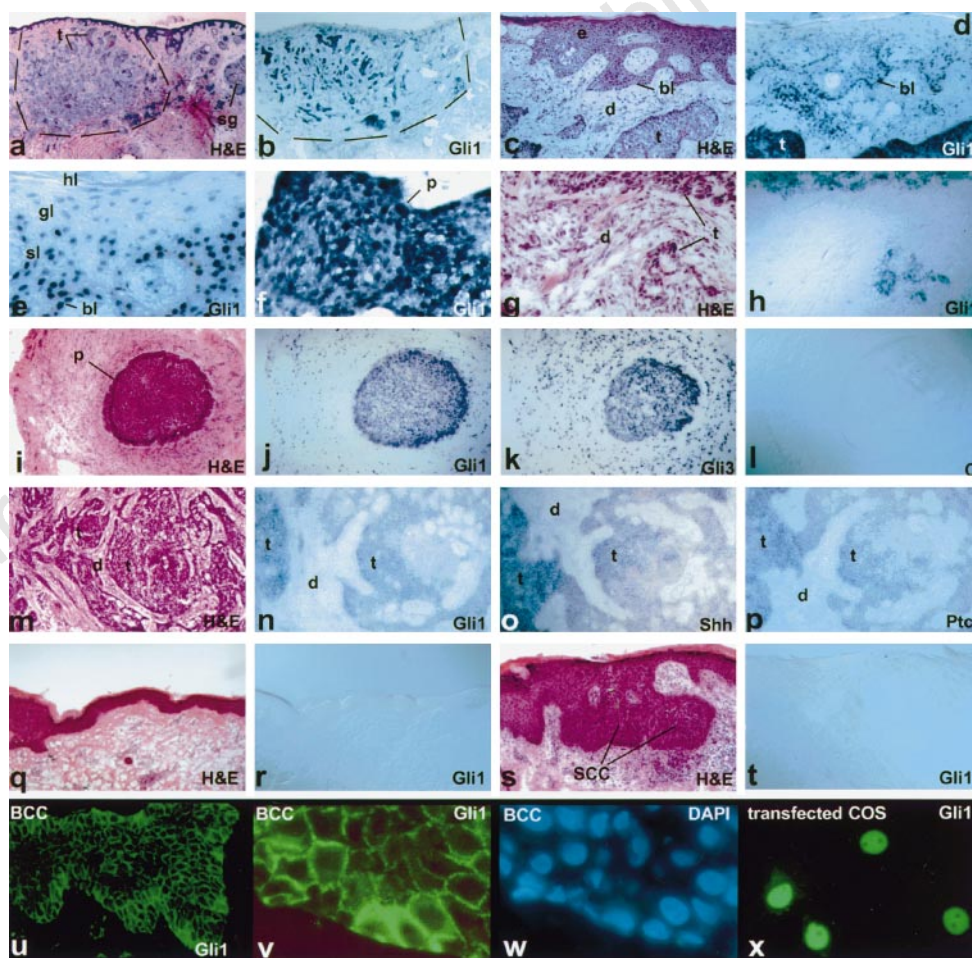


Figure 2 Gene expression in basal cell carcinomas and cell lines. **a–p**, Sections of BCC excisions showing the distribution of tumour masses as seen by histological staining (**a, c, g, i, m**), *Gli1* mRNA (**b, d, e, f, h, j, n**), *Gli3* mRNA (**k**), *Shh* mRNA (**o**) or *Ptc* mRNA (**p**). As a control, absence of label is seen after hybridization with a *Gli1* sense probe (**l**). Matched samples from the same specimens are shown in **a** and **b**, **c** and **d**, **g** and **h**, **i** to **l**, and **m** to **p**. **e, f**, Details of the specimen shown in **a–d**, respectively. **q, r**, Sections of normal skin distal from tumorigenic regions in a BCC excision showing the absence of *Gli1* expression. **s, t**, Sections of an excised sample of squamous cell carcinoma (SCC) *in situ* showing the absence of *Gli1* expression. **u–x**, Labelling

of excised BCC sections with affinity-purified anti-human *Gli1* antibodies (**u, v, x**) or with the DNA-binding dye DAPI showing the position of nuclei (blue in **w**). **x**, Expression of nuclear *Gli1* protein in COS-7 cells transfected with plasmids driving the expression of the human glioma *Gli1* cDNA. H&E, haematoxylin and eosin stain; bl, basal layer; d, dermis; e, epidermis; gl, granular layer; hl, horny layer; p, pallisade in the periphery of the tumour nodule; sl, spiny layer; t, tumour. In all cases the skin surface is up except in **i–l** in which it is to the left. **a–f**, case no. 5; **g, h**, case no. 7; **i–l**, case no. 12; **m–p**, case no. 61; **q, r**, a normal skin region of case no. 18; **s, t**, case no. 15; all as listed in Table 1.

Table 1 Gene expression in human skin tumours

Case	type	location	Gli1 ab	HNF-3 β Ab	Gli1-as	Gli1-s	Gli3-as	Gli3-s	Shh-as	Shh-s	Ptc-as	H&E
1	BCC	auricular	+									+
2	BCC	nasolabial fold	+	-								+
3	BCC	temple	+	-								+
4	BCC	forehead	+									+
5	BCC	post-auricular			++							+
6	BCC	inner canthus	+	-	++							+
7	BCC	post-auricular	+	-	++	-						+
8	BCC	nasolabial fold			++							+
9	BCC	post-auricular			++							+
10	BCC	canthus			+/-	-	+/-					+
11	BCC	canthus			+	-	+					+
12	BCC	back			++	-	++	-				+
13	BCC	nasal rim			++	-		-				+
14	BCC	nasal rim			++	-	-	-				+
17	BCC	nasal rim			+/-	-	-	-				+
18	BCC	nasal rim			++	-	-	-				+
24	BCC	nose			+	-	-		+			+
26	BCC	periareolar			++		+		++	-		+
27	BCC	eyelid			+		-		-	-		+
28	BCC	nose			+		+/-	-				+
29	BCC	temple			+		+		+	-		+
30	BCC	midback			++		+		+	-		+
32	BCC	lat. forehead			+		-		-			+
33	BCC	eyebrow			+		+		+			+
34	BCC	nosetip			+		+		-			+
37	BCC	lat. upper cheek			+/-		+/-		+/-			+
39	BCC	upper lip			+		+		+/-			+
41	BCC	malar ocular			++		++		+/-			+
42	BCC	malar ocular			++		+		-			+
43	BCC	temple			++		+		-			+
44	BCC	nose			+		+		-			+
45	BCC	cheek			++		+		+			+
46	BCC	nostril			++		++		-			+
47	BCC	zygoma			++		+		-			+
48	BCC	upper eyelid			+		++		+			+
51	BCC	glabella			++		+		+			+
52	BCC	nose			+		++		-			+
53	BCC	nose			+		+/-		-			+
54	BCC	ear			++		+/-		+/-			+
57	BCC	clavicle			+		-		-			+
59	BCC	nose			++				+	-	++	+
61	BCC	nose			++				++	-	+	+
63	BCC	nose			+						++	+
64	BCC	forehead			+/-		-		-	-	++	+
66	BCC	temple			+				-	-	+	+
67	BCC	forehead			+				+	-	++	+
69	BCC	forehead			++				-	-	++	+
70	BCC	scalp			++				-	-	++	+
71	BCC	eye			+				-	-	+	+
72	BCC	nose			++				-	-	++	+
74	BCC	temple			+				-	-	+	+
15	SCC	upper back			-	-	-	-				+
23	SCC	preauricular			-		-		-	-		+
25	SCC	eyelid			-		-		-	-		+
31	SCC	elbow			-		-		-			+
35	SCC	cheek			-		-		-			+
40	SCC	cheek			-		-		-			+
49	SCC	cheek			-		-		-	-		+
55	SCC	hand			-		+/-		-	-		+
56	SCC	neck			-		-		-	-		+
58	SCC	bridge nose			-		-		-	-		+

The case number, type of tumour (BCC or SCC *in situ*) and location of the tumour is given on the left. The presence (+) or absence (-) of gene expression is indicated, with strong expression indicated by ++. A section of each excision was also stained with haematoxylin and eosin (H&E) for histological examination and confirmation of the presence of tumour. Case no. 10 was ambiguous and was counted as negative for *Gli1* expression. Abbreviations: as, antisense RNA probe; s, sense RNA probe; Ab, antibody; lat., lateral.

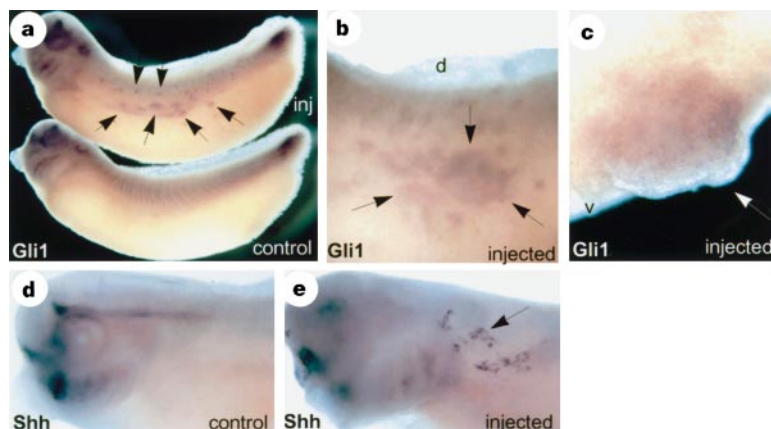


Figure 3 Expression of endogenous *Gli1* and *Shh* in *Gli1*-induced tadpole tumours. Frog tadpoles (stages 34-36) injected with human *Gli1* plasmids (**a-c**) or synthetic frog *Gli1* RNA (**e**), but not control embryos (**a** bottom **d**), show ectopic expression of endogenous *Gli1* (arrows in **a** top, **b**, **c**) or *Shh* (arrow in **e**). Human and frog *Gli1* do not cross-hybridize *in situ* hybridization. *Gli1* is normally expressed in several tissues including the neural tube but not the early epidermis¹¹. *Shh* is normally expressed in the nervous system and head structures including the branchial arches⁵. Anterior is to the left and dorsal side is up. Embryos were not cleared.

Interfollicular human basal cells and the early tadpole epidermis do not normally express *Gli1* genes, and the reason why the epidermis is responsive to Shh and *Gli1* is not clear. However, we detected expression of *Gli1* and *Shh* in human hair follicles, and *Shh*, *Gli1*, *Gli2* and *Gli3* were also expressed in mouse hair follicles^{14,21,22} during the growing phases with highest expression of *Shh* and *Gli1* in matrix keratinocytes of the bulb (not shown). This indicates that normal epidermal development involves the selective activation of the Shh signalling pathway during follicular formation and provides a context for the ability of embryonic and non-follicular-basal epidermal cells to respond to overexpression of *Gli1*.

Gli1 has been found to be amplified only in a small number of gliomas and other tumours^{6,24,25}. The high incidence of *Gli1* expression in BCCs contrasts with the relatively infrequent occurrence of other oncogenes, such as mutated *ras* alleles²³. Because hair follicles normally activate the Shh signalling pathway during growth, BCCs could derive from the neoplastic transformation of these cells. Indeed, BCCs show traits of follicular differentiation²⁶, including the expression of *Gli3*. It is possible, however, that BCCs derive from non-follicular basal cells which express *Gli1* ectopically. In this case, the normal interaction taking place between the dermal papilla and the hair bulb could be activated inappropriately in non-follicular basal cells, resulting in the activation of the Shh signalling pathway and formation of BCCs. We propose that any mutations that activate the Shh signalling pathway will lead to ectopic *Gli1* expression and BCC formation. In familial BCCs showing loss of *Ptc* function^{1,2}, we predict that *Gli1* will be ectopically expressed. However, mutations in *Ptc* cannot account entirely for *Gli1* activation. We found that *Gli1* and *Ptc* are consistently expressed in sporadic BCCs, whereas in other studies only a fraction of sporadic BCCs showed an altered *Ptc* allele^{2,3}. Because there may be a regulatory loop in the Shh signalling pathway, *Gli1* expression would appear to be both a cause and an effect of BCC development.

Ectopic expression of Shh in basal cells of transgenic mice has recently been shown to result in the development of BCC-like tumours⁴. However, the inability of Shh to induce tumour formation in the tadpole epidermis and its inconsistent expression in BCCs and tadpole tumours raise the possibility that normally there may be restrictions to the induction and action of Shh in epidermis similar to those present in the neural plate¹¹. Such restrictions could prevent BCC formation adjacent to follicle cells expressing Shh during normal hair growth and after plucking, and the uncontrolled spread of BCCs throughout the surrounding tissue when the tumours induce Shh. Independent of whether Shh can initiate BCC formation, its expression in BCCs suggests a mode of autocrine tumour maintenance as secreted Shh from the tumour cells could activate its signalling pathway, leading to new expression of *Gli1*. Activation of autocrine Shh signalling could underlie the formation of persistent epidermal tumours in embryos that transiently expressed *Gli1* through microinjection. However, transcription of endogenous *Gli1* but not *Shh* was always detected in *Gli1*-induced tumours and BCCs, suggesting that a *Gli1* regulatory loop operating downstream of Shh is functional.

The recurrence of BCCs at sites adjacent to previous tumours could result from the observed ectopic expression of *Gli1* in basal cells in a wide region extending beyond the neoplastic sites. This raises the possibility that *Gli1* expression in basal cells is an early event and could be used as a diagnostic tool. Finally, therapeutic agents for BCCs are likely to include inhibitors of the Shh signalling pathway and *Gli1* function. □

Methods

Embryos and microinjection. *Xenopus laevis* embryos were obtained and manipulated by standard procedures²⁷. Microinjections were performed into the animal-most region of one cell at the two-cell stage to bias the distribution of the injected plasmids or RNAs to the ectoderm and to have half of the embryo as an undisturbed internal control. Plasmid DNA (200 pg) or synthetic

RNAs (2 ng) made by *in vitro* transcription were delivered by microinjection. Frog *Gli1*, human *Gli1* and *Gli3* plasmids were as described⁵.

In situ hybridization, immunocytochemistry, histology and cell lines. Frog embryos were processed for *in situ* hybridization with digoxigenin-labelled RNA probes²⁸. Frog *Gli1* and *Shh* plasmids to make sense or antisense RNA probes were as described⁵. Frozen cryostat sections of tumour specimens excised by the Mohs technique (by P.R.) were processed by *in situ* hybridization with digoxigenin-labelled RNA probes²⁹. Plasmids with human *Gli1* and *Gli3* cDNAs^{6,7} and mouse *Shh* and *Gli1-3* cDNAs used to make sense and anti-sense RNA probes were as described⁵. The human *Shh* probes were made from a plasmid subclone of a 409-base pair RT-PCR product or from a human cDNA¹⁹. Immunocytochemistry with anti-human *Gli1* affinity-purified polyclonal antibodies⁵, anti-frog HNF-3 β or anti-rat HNF-3 β polyclonal antibodies^{11,16} were performed in whole-mount labelling or in 5–15 μ m cryostat sections. Nuclei were visualized by staining with the DNA-binding dye DAPI after antibody incubations. Histological sections of injected tadpoles were obtained by cutting paraplast-embedded samples in a microtome⁵. These sections and one section of each tumour sample were also stained with haematoxylin and eosin for histological and pathological examination (by P.H.). β -Galactosidase activity was revealed by the X-gal reaction. COS-7 cells were obtained from ATCC and cultured under the specified conditions. Transfections were performed with lipofectamine (Gibco-BRL) as specified by the manufacturer. Cells were assayed 24–48 h after transfection.

RNA isolation and RT-PCR. RNA from frozen excisions was extracted by the guanidinium isothiocyanate, acid phenol method. cDNA was made with random hexamers and BRL Superscript reverse transcriptase. PCR was performed at 57°C for 40 cycles with the following primers to human *Gli1*: *Gli1*-U, CAGAGAATGGAGCATCCTCC; and *Gli1*-D, TTCTGGCTCTTCCTGTAGCC, yielding a 412-bp product. Human *Gli3*: *Gli3*-U, GCAGCCACAG AATGTCC; and *Gli3*-D, AGGGATATCCAATCGAGGAATCG, yielding a 293-bp product. Human *Shh*: *Shh*-U2, GAAGATCTCCAGAACTCC; and *Shh*-D, TCGTAGTGCAGAGACTCC, yielding a 233-bp product. Mouse *S17*, which works well with human cDNA: *S17*-U, GCTATGTCACGCATCTGATG; and *S17*-D, CCTCAATGATCTCCTGATC, yielding a 137-bp product. Human *Ptc*: *Ptc*-U, GAATCCAGGCATCACCACC; and *Ptc*-D, CCACGTCCTGCAGCTC AATG, yielding a 490-bp product. The RT-PCR *shh* clone used to make RNA probes derived from a reaction using *Shh*-U1, AGATGTCTGCTGCTAGTCC, and *Shh*-D. *Shh* RNA probes were also made from a large human cDNA¹⁹.

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A SNARE involved in protein transport through the Golgi apparatus

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In eukaryotic cells, the Golgi apparatus receives newly synthesized proteins from the endoplasmic reticulum (ER) and delivers them after covalent modification to their destination in the cell. These proteins move from the inside (*cis*) face to the plasma-membrane side (*trans*) of the Golgi, through a stack of cisternae, towards the *trans*-Golgi network (TGN), but very little is known about how proteins are moved through the Golgi compartments. In a model known as the maturation model^{1–3}, no special transport process was considered necessary, with protein movement along the Golgi being achieved by maturation of the cisternae. Alternatively, proteins could be transported by vesicles^{4–6} or membrane tubules^{7,8}. Although little is known about membrane-tubule-mediated transport^{7,8}, the molecular mechanism for vesicle-mediated transport is quite well understood, occurring through docking of SNAREs on the vesicle with those on the target membrane^{4–6,9–13}. We have now identified a protein of relative molecular mass 27K which is associated with the Golgi apparatus. The cytoplasmic domain of this protein or antibodies raised against it quantitatively inhibit transport *in vitro* from the ER to the *trans*-Golgi/TGN, acting at a stage between the *cis*/medial- and the *trans*-Golgi/TGN. This protein, which behaves like a SNARE and has been named GS27 (for Golgi SNARE of 27K), is identical to membrin, a protein implicated earlier in ER-to-Golgi transport¹⁴. Our results suggest that protein movement from medial- to the *trans*-Golgi/TGN depends on SNARE-mediated vesicular transport.

Database searches using a *Caenorhabditis elegans* protein sequence (accession number P41941) that is weakly related to that

of the yeast protein Bos1p, a v-SNARE involved in ER–Golgi transport^{15–17}, led to the identification of expressed-sequence tags (ESTs) encoding the potential human (accession number T88746) and mouse (accession numbers AA165867, W75416 and W30385) counterparts. Rat complementary DNAs were isolated by screening

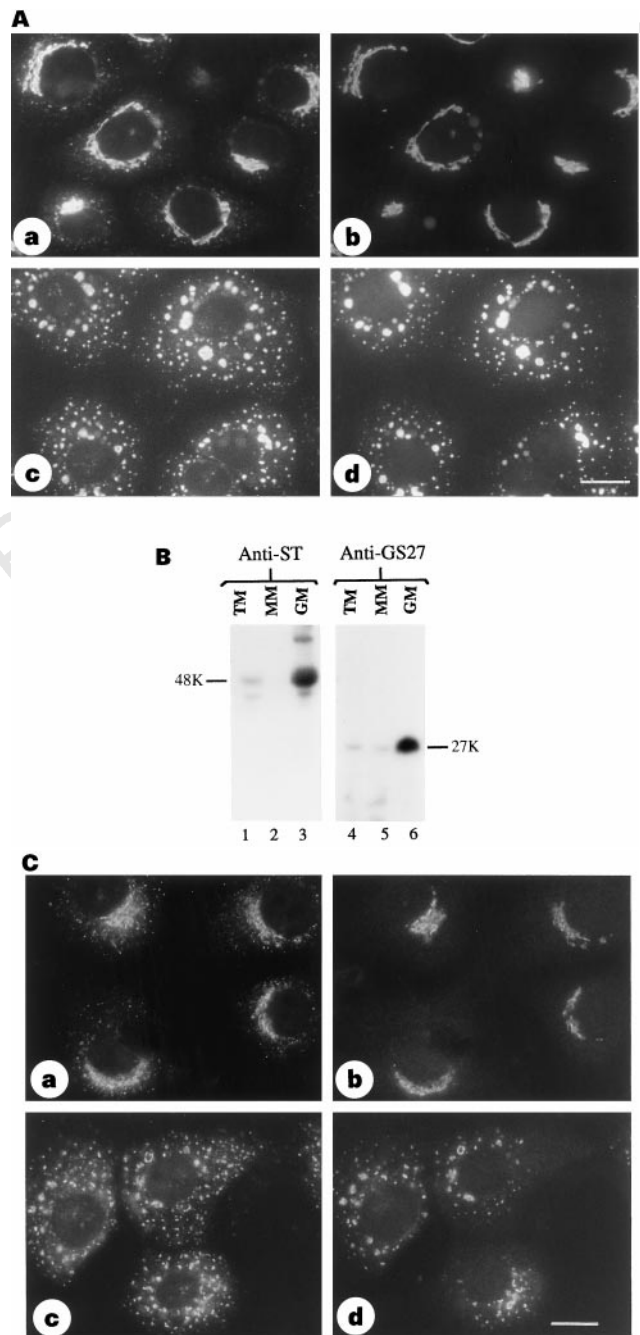


Figure 1 **A**, HA-epitope-tagged GS27/membrin is associated with the Golgi apparatus and vesicular structures. HA-GS27 (**a** and **c**) and Golgi mannosidase II (**b** and **d**) were double-labelled in control (**a**, **b**) and nocodazole-treated (**c**, **d**) cells. HA-GS27 was co-localized with mannosidase II in the control and in fragmented Golgi. The vesicular structures marked by GS27/membrin were devoid of mannosidase II labelling. **B**, Enrichment of GS27/membrin in the Golgi membrane. Proteins of total membranes (TM), microsomal membranes (MM) and Golgi-enriched membranes (GM) were analysed by immunoblot analysis using antibodies against α 2, 6-sialyltransferase (ST) (lanes 1–3) or antibodies against GS27/membrin (lanes 4–6). **C**, Endogenous GS27/membrin is associated with the Golgi apparatus and its vesicular structures. Control (**a**, **b**) or nocodazole-treated (**c**, **d**) cells were double-labelled with antibodies against GS27/membrin (**a** and **c**) or Golgi mannosidase II (**b** and **d**).

a rat brain cDNA library¹⁸. The corresponding protein was named GS27 because of its function and size; GS27 is identical to membrin¹⁴, a protein identified independently in a syntaxin 5-containing complex which was proposed to be primarily associated with the ER and 'involved' in ER–Golgi vesicular transport. We now provide evidence for a different cellular and mechanistic function for GS27/membrin.

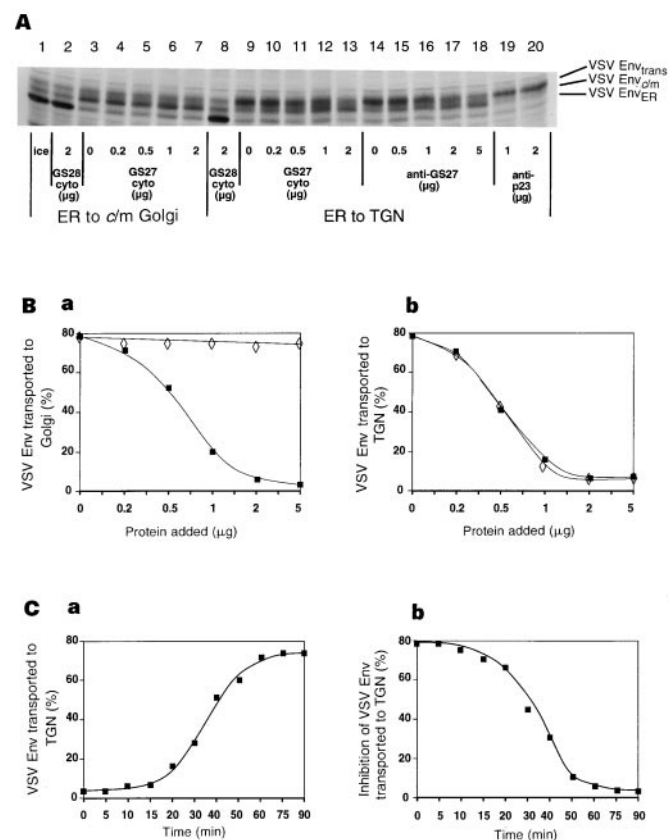


Figure 2 **A**, Recombinant cytoplasmic (cyto) domain of GS27/membrin and its antibodies specifically inhibit the conversion of VSV Env from the *cis*/medial-Golgi form (VSV Env_{c/m}) into the *trans*-Golgi/TGN form (VSV Env_{trans}) but have no effect on conversion of VSV Env from the ER form (VSV Env_{ER}) into VSV Env_{c/m}. *In vitro* ER-to-*cis*/medial-Golgi (lanes 1–7) or ER-to-*trans*-Golgi/TGN assays (lanes 8–20) were supplemented with the indicated materials and endo H-digested VSV Env was analysed. **B**, Dose-dependent effects of GS28c (squares) and GS27c (diamonds) on *in vitro* ER-to-*cis*/medial-Golgi (**a**) and ER-to-*trans*-Golgi/TGN (**b**) transport. *In vitro* transport was assayed in the presence of increasing amounts of GS27c or of GS28c; transport reactions were treated with endo H and resolved by SDS-PAGE. The intensities of bands in each lane, representing VSV Env_{trans}, Env_{c/m} and Env_{ER}, were quantified by phosphorimaging and the sum of these three forms was defined as 100% (total Env). For ER-to-Golgi transport, the sum of intensities (×100) for Env_{trans} and Env_{c/m} over that of total Env was defined as the percentage transported to the Golgi (**a**). For transport to the *trans*-Golgi/TGN, the intensities (×100) for Env_{trans} over that of total Env was defined as the percentage transported to the TGN (**b**). Averages of three independent experiments are presented. **C**, GS27c can be added to the *in vitro* ER-to-*trans*-Golgi/TGN assay at relatively late stages to achieve significant inhibition. **a**, Kinetics of conversion of VSV Env_{ER} to VSV Env_{trans}. Maximal conversion was achieved within 60–90 min. **b**, Effects of GS27c on ER-to-*trans*-Golgi/TGN transport when added to the transport assay at different periods of time of the transport reaction. For example, after 30 min in the transport assay, about 30% of Env was converted to VSV Env_{trans} (**a**). When GS27c was added at this time and the transport reaction resumed, about 50% of total Env could still be prevented from being transported to the *trans*-Golgi/TGN (**b**).

We found that an N-terminal epitope-tagged form of GS27/membrin (haemagglutinin(HA)-GS27) was associated with the Golgi apparatus and its peripheral vesicular structures (Fig. 1A), so we investigated the localization of endogenous GS27/membrin. The cytoplasmic domain (GS27c) of rat GS27/membrin (residues 1–190) was produced in bacteria with a C-terminal His × 6 tag and the purified protein was used to prepare polyclonal antibodies against GS27/membrin. As shown in Fig. 1B, GS27/membrin antibodies reacted specifically with a 27K protein in Golgi membranes where it was enriched. Endogenous GS27/membrin was associated with the Golgi apparatus and its surrounding vesicular structures (Fig. 1C).

To determine a possible function for GS27/membrin, we used a well established *in vitro* system that reconstitutes vesicular transport of the envelope glycoprotein (Env) of vesicular stomatitis virus (VSV ts045) from the ER to either the *cis*/medial-Golgi or the *trans*-Golgi/TGN^{19–21} in perforated NRK cells. The ER-to-*cis*/medial-Golgi assay monitors vesicular transport through the conversion of VSV Env from the endoglycosidase(endo)-H sensitive to the resistant form; vesicular transport from the ER to *trans*-Golgi/TGN is measured by the conversion of Env into forms that are not only endoH-resistant but have also incorporated galactose and sialic acids (events that are specific for the *trans*-Golgi/TGN). As shown in Fig. 2A, GS27c had no effect on transport from the ER to the *cis*/medial-Golgi, regardless of the amount added to the transport reaction (lanes 3–7), whereas this transport event was quantitatively inhibited (lane 2) by the cytoplasmic domain (GS28c) of a previously identified Golgi SNARE (GS28) known to be involved in transport from the ER to the *cis*/medial-Golgi^{22,23}. These results suggest that, unlike GS28, GS27/membrin does not play a role in transport from the ER to the *cis*/medial-Golgi. When GS27c was added to the *in vitro* ER-to-*trans*-Golgi/TGN assays, it inhibited the appearance of the *trans*-Golgi/TGN form in a dose-dependent way, with a corresponding increase in the *cis*/medial-Golgi form (Fig. 2A, lanes 9–13). In addition, purified polyclonal antibodies against GS27/membrin also caused dose-dependent inhibition of transport from the ER to the *trans*-Golgi/TGN at the same stage (Fig. 2A, lanes 14–18). The quantitative and selective inhibition of conversion of Env from the *cis*/medial-Golgi to the *trans*-Golgi/TGN form by GS27c and the antibodies suggests that GS27/membrin is critical for the transport from the *cis*/medial- to the *trans*-Golgi/TGN but not for transport from the ER to the *cis*/medial-Golgi. Under identical conditions, GS28c inhibited the appearance of both *cis*/medial-Golgi and *trans*-Golgi/TGN forms in the ER-to-*trans*-Golgi/TGN transport assay (Fig. 2A, lane 8), consistent with its role in transport from the ER to the *cis*/medial-Golgi.

The dose-dependent effects of GS27c and GS28c on *in vitro* ER-to-*cis*/medial-Golgi transport and ER-to-*trans*-Golgi/TGN transport are shown in Fig. 2B: the dose response of inhibition by GS28c (filled squares) of ER-to-*cis*/medial-Golgi (Fig. 2B, a) and of ER-to-*trans*-Golgi/TGN (Fig. 2B, b) transport is similar, with inhibition being almost complete at ≥1.5 μg GS28c. Again, GS27c (Fig. 2B, open diamonds) had no effect on ER to *cis*/medial-Golgi transport (Fig. 2B, a) but gave a similar dose–response curve to GS28c on ER-to-*trans*-Golgi/TGN transport (Fig. 2B, b) but with most Env being arrested in the *cis*/medial-Golgi form when ≥1.5 μg GS27c was added. Figure 2C, a shows the kinetics of *in vitro* transport from the ER to the *trans*-Golgi/TGN with maximal transport being achieved in 60–90 min. We did time-course experiments (Fig. 2C, b), in which ER-to-*trans*-Golgi/TGN transport was assayed for increasing times at 32 °C, after which GS27c was added at 4 °C and incubation continued at 32 °C: these showed that GS27c could be added relatively late on and still be inhibitory, suggesting that inhibition of ER-to-*trans*-Golgi/TGN transport by GS27c occurs at a late stage and that the action of GS27c is direct.

Using a morphological assay²⁴, we found that GS27c and the antibodies had no effect on the movement of Env from the ER to the

Golgi, as indicated by the Golgi marker mannosidase II, but that transport out of the Golgi was inhibited (data not shown), consistent with the results of the biochemical assay. Taken together, our results suggest that GS27/membrin may participate in a transport event from the *cis*/medial- to the *trans*-Golgi/TGN, although it is possible that GS27/membrin is involved in intra-Golgi retrograde transport that is tightly coupled to anterograde transport from the *cis*/medial- to the *trans*-Golgi/TGN.

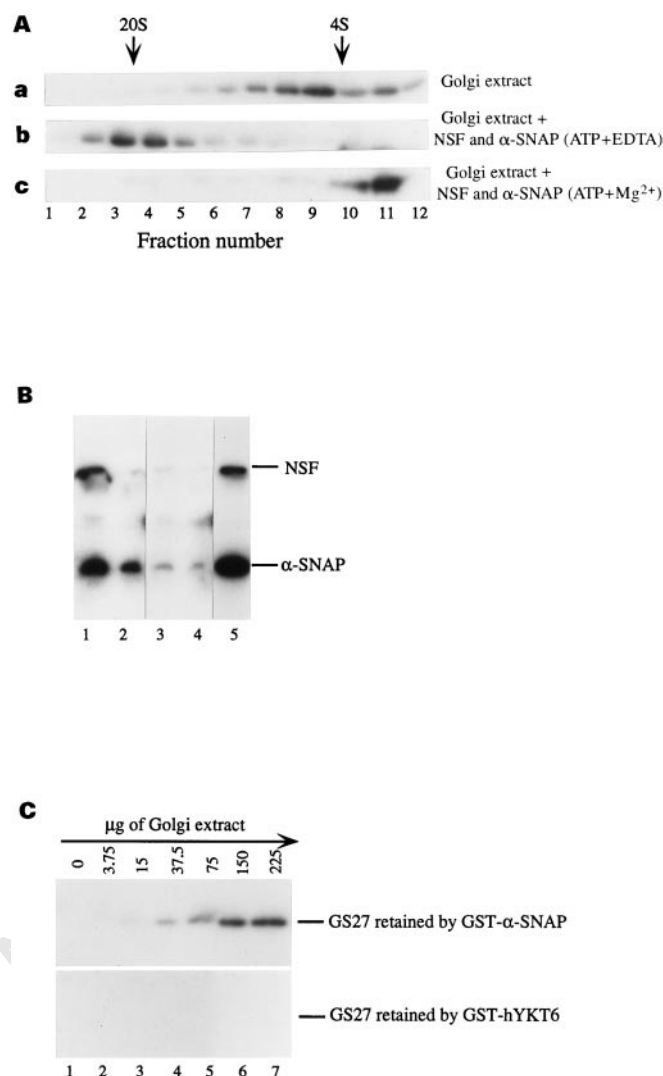


Figure 3 GS27/membrin is a SNARE. **A**, Golgi extract alone (top), or supplemented with NSF and α -SNAP (bottom two rows) was analysed in assembly (top two rows) or disassembly (bottom) buffer by glycerol-gradient centrifugation. Fractions were collected from the bottom of the centrifuge tubes and analysed by immunoblotting using antibodies against GS27/membrin. The positions of 20S and 4S fractions are indicated. **B**, GS27/membrin-containing 20S fractions were combined and immunoprecipitated with antibodies against GS27/membrin (lanes 1, 2) or control antibodies (lanes 3, 4). Immunoprecipitates were eluted in disassembly (lanes 1 and 3) or assembly (lanes 2 and 4) buffer. The eluates, together with 100 ng recombinant NSF and α -SNAP (lane 5), were analysed by immunoblotting using antibodies against NSF and α -SNAP. NSF and α -SNAP were specifically eluted from GS27/membrin immunoprecipitates under conditions that promoted the disassembly of the 20S SNARE complex (lane 1). **C**, GS27/membrin from the Golgi extract was specifically retained by immobilized GST- α -SNAP but not by GST-hYKT6. The indicated amounts of Golgi extract were incubated with 2 μ g GST- α -SNAP or GST-hYKT6 immobilized on beads. GS27/membrin retained by the beads was analysed by immunoblotting.

To understand how GS27/membrin is involved in transport from the *cis*/medial- to the *trans*-Golgi/TGN, we tested whether it could function as a SNARE. GS27/membrin was found to sediment at $\sim$ 6S (Fig. 3A, top row) when rat-liver Golgi extracts were analysed in sedimentation gradients. GS27/membrin was shifted to the 20S fraction in the presence of both NSF (*N*-ethylmaleimide-sensitive fusion protein) and α -SNAP (soluble NSF attachment protein) under conditions that promoted assembly of the 20S SNARE complex^{11,23,25} (Fig. 3A, middle row), but not under conditions favouring disassembly of the SNARE complex (Fig. 3A, bottom row). Under disassembly conditions, GS27/membrin sedimented at 3S, indicating that any association of GS27/membrin in the Golgi extract with other proteins has been disrupted. When GS27/membrin-containing 20S fractions were immunoprecipitated by specific antibodies, both NSF and α -SNAP were released from the precipitated complex under conditions that trigger ATP hydrolysis by NSF and disassembly of the SNARE complex (Fig. 3B, lane 1), but not under conditions that prevent ATP hydrolysis by NSF (Fig. 3B, lane 2). We also found that GS27/membrin in the Golgi extract could interact specifically with a glutathione-S-transferase (GST)- α -SNAP fusion protein immobilized on beads (Fig. 3C). Under identical conditions, GS27/membrin did not interact with immobilized GST-hYKT6 (a human counterpart of the yeast SNARE Ykt6 (refs 26, 27) fused to the C terminus of GST) (Fig. 3C) or with GST itself (data not shown). Our results indicate that protein movement from the medial- to the *trans*-Golgi/TGN is dependent on a SNARE-mediated vesicular transport event. The identification of GS27/membrin as a SNARE and our demonstration of its role in this transport event should enable the molecular mechanism of this poorly characterized intra-Golgi transport step to be clarified. □

Methods

cDNA cloning of rat GS27/membrin. Human GS27/membrin cDNA was radiolabelled with ³²P-dCTP (oligolabelling kit from Pharmacia) and used to screen a rat brain cDNA library (2×10^6 PFU) in the Zap II vector (Stratagene) essentially as described¹⁸.

Epitope tagging and transfection. The entire coding region of human GS27/membrin, together with sequences encoding a haemagglutinin (HA) tag at the 5' end immediately downstream of the initiating methionine, was subcloned using PCR into the pCI-neo expression vector (Promega) by engineering *Eco*RI and *Sal*I sites at the 5' and 3' ends, respectively. The 5' primer encompassing the HA tag used was 5'-CCGGAATTCCACCATGAATTATCCGTATGATC-TACCGGATTATGCTGATCCCCTGTTCCAGCAAACGCAC, and the nucleotide sequence 5'GCGCGGTCGACTCATGTCAGGTACTGCACCACGAG was used as the 3' primer. Plasmids were transfected into NRK cells using lipofectamine reagent (Gibco BRL). One clone designated 31G was processed for further study.

Expression and purification of recombinant GS27c, GST-GS27c, GST- α -SNAP, GST-hYKT6, NSF and α -SNAP. For production of recombinant glutathione-S-transferase fusion proteins, the coding region of GS27/membrin without the transmembrane domain (GS27c; 1–570 bp) was subcloned into the bacterial expression vector pGEX-KG (ref. 28). Unique *Eco*RI and *Xho*I sites were engineered into the GS27/membrin cDNA at the start and stop codon, respectively, using synthetic oligonucleotides (5'-CCGGAATTCTAATG-GAGCCACTATACCAACAAACG for *Eco*RI and 5'-CCCGCTCGAGTCAC-TTGTCCTCCAAGGCCGCTT for *Xho*I). The cDNA insert was generated by PCR and ligated to pGEX-KG. For the GST- α -SNAP construct, the primers were 5'GTGAATTCTAATGGACAACCTCCGGGAAGGAGCG for *Eco*RI, and 5'GTCTCGAGTTAGCGCAGGTCTTCTCTCGTCACCCTG for *Xho*I. Ligated plasmids were transfected into DH5 α cells and ampicillin-resistant colonies expressing the GST-GS27 fusion protein were screened. GST fusion proteins were purified as described^{16,18}. For preparation of His $\times$ 6 tagged proteins, the GS27c region was subcloned into the pQE60 expression vector (Qiagen) using *Nco*I and *Bam*HI sites engineered at the 5' and 3' ends respectively. Primer sequences were 5'-CTCTCCATGGAGCCACTATACCAACAAAC for *Nco*I and 5'-CTCTGGATCCCTTGTCCTCCAAGGCCGCTT for *Bam*HI. The ligated

plasmid was transformed into XL-1 blue cells and plasmid DNA from colonies harbouring the construct was retransformed into M15 cells. Transformants were screened for recombinant protein production and cultures grown and induced as described for GST fusion proteins. His $\times$ 6-tagged proteins were purified as described²³. GST-hYKT6 was similarly produced and purified.

Preparation and affinity-purification of antibodies against GS27/membrin. For preparation of polyclonal antibodies against GS27/membrin, 400 μ g GS27c was emulsified in complete Freund's adjuvant and injected subcutaneously into rabbits. Booster injections containing a similar amount of antigen emulsified in incomplete Freund's adjuvant were given after 2, 4 and 7 weeks, respectively. Rabbits were bled 10 d after the corresponding booster injection. Affinity purification of monospecific antibodies was achieved by using the GST-GS27c fusion protein coupled to cyanogen bromide-activated Sepharose (2 mg protein per ml Sepharose beads).

Indirect immunofluorescence microscopy, SDS-PAGE and immunoblot analysis. These have all been described¹⁸.

In vitro ER-Golgi transport. The transport of VSV Env from the ER to the Golgi complex was assayed as described^{19–21}. Briefly, NRK cells were grown on 10-cm Petri dishes to form a confluent monolayer and were then infected with the temperature-sensitive strain VSVt045. Cells were pulse-labelled with ³⁵S-methionine at the restrictive temperature and mechanically permeabilized on ice. These semi-intact cells were then incubated in a complete assay cocktail (40 μ l) containing (final concentrations) 25 mM HEPES-KOH, pH 7.2, 90 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, 1.8 mM CaCl₂, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine phosphokinase, 25 μ g rat liver cytosol and 5 μ l ($\sim 1\text{--}2 \times 10^5$ cells) of semi-intact cells. Additional reagents were added as indicated. Samples were incubated for 60 min at 32°C and transport terminated by transfer to ice. Membranes were collected by a brief spin, solubilized in 0.1% SDS and digested overnight with endo H. Subsequently, 5 $\times$ concentrated gel-sample buffer was added and samples were separated on 7.5% SDS-polyacrylamide gels. Transport was quantified using a Phosphor Imager (Molecular Dynamics). To investigate transport of the VSV Env protein from the ER to the TGN, the transport cocktail was supplemented with (final concentration) 100 μ M UDP-N-acetylglucosamine (UDP-GlcNAc), 200 μ M UDP-galactose (UDP-Gal) and 100 μ M CMP-sialic acid (CMP-SA) and the incubation time extended to 90 min. The morphological assay has been described²⁴.

SNARE-complex formation. The formation of the 20S SNARE complex has been described^{11,23,25}.

In vitro protein interaction assay. Golgi-enriched membranes (1 mg) were extracted in 500 μ l incubation buffer (100 mM KCl, 20 mM HEPES, pH 7.3, 2 mM EDTA, 2 mM DTT) containing 1% Triton X-100 and incubated at 4°C for 1 h, with agitation. Extracted Golgi membranes were diluted with 500 μ l incubation buffer without Triton X-100 and then centrifuged at 100,000g at 4°C for 30 min. Beads containing 2 μ g GST- α -SNAP, GST-hYKT6 or GST were washed twice with incubation buffer containing 0.5% Triton X-100 (1 ml each) before incubating with different amounts of extracted Golgi proteins in 100 μ l at 4°C for 1 h with agitation. Beads were then washed three times with incubation buffer containing 1% Triton X-100, once with incubation buffer containing 0.1% Triton X-100, and then processed for immunoblot analysis to detect GS27/membrin.

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Correspondence and requests for materials should be addressed to W.H. (e-mail: mcbhwj@leonis.nus.sg). The nucleotide and amino-acid sequences of human, rat and mouse GS27/membrin have been deposited with GenBank under accession numbers AF007548, AF007549 and AF007550, respectively.

Crystallographic structure of the T domain–DNA complex of the *Brachyury* transcription factor

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The mouse *Brachyury* (*T*) gene is the prototype of a growing family of so-called T-box genes which encode transcriptional regulators and have been identified in a variety of invertebrates and vertebrates, including humans^{1–6}. Mutations in *Brachyury* and other T-box genes result in drastic embryonic phenotypes, indicating that T-box gene products are essential in tissue specification, morphogenesis and organogenesis^{7–11}. The T-box encodes a DNA-binding domain of about 180 amino-acid residues, the T domain¹². Here we report the X-ray structure of the T domain from *Xenopus laevis*² in complex with a 24-nucleotide palindromic DNA duplex. We show that the protein is bound as a dimer, interacting with the major and the minor grooves of the DNA. A new type of specific DNA contact is seen, in which a carboxy-terminal helix is deeply embedded into an enlarged minor groove without bending the DNA. Hydrophobic interactions and an unusual main-chain carbonyl contact to a guanine account for sequence-specific recognition in the minor groove by this helix. Thus the structure of this T domain complex with DNA reveals a new way in which a protein can recognize DNA.

DNA target sites of T protein were isolated *in vitro* by nucleotide selection from a random pool of oligonucleotides¹². A 20-base-pair

(bp) binding site with an almost perfect palindromic sequence was defined as the consensus sequence. Residues 1–226 of the T protein from *X. laevis* were bacterially expressed and co-crystallized with a 24-nucleotide DNA duplex containing a palindromic binding site. The structure of the complex was solved at 2.5 Å resolution by multiple isomorphous replacement using three iodinated DNA derivatives and one selenomethionine derivative. This structure contains residues 39–222 of monomer I (and residues 39–221 of monomer II) and the 24-nucleotide DNA duplex. The amino-terminal residues, which are not part of the T-domain consensus, and the remaining carboxy-terminal residues are disordered (Fig. 1).

The X-ray structure shows that the T domain is bound as a dimer to the DNA, with the dimer interface lying above the minor groove (Fig. 2). The dimer forms a large arc which spans the DNA and allows it to contact its 20-bp DNA-recognition sequence. As a result, a huge hole of almost 10 Å diameter is formed between the dimer interface and the contact regions of each monomer with its DNA half-site.

The core of each monomer consists of a seven-stranded β -barrel related to an immunoglobulin fold. Strands A, B and E form one sheet, whereas strands C, C', F and G form the other sheet of the β -barrel. Strand D, commonly found in immunoglobulin constant domains, is replaced by helix H2 (Fig. 1). One end of the β -barrel forms the dimer interface. At the other end, which points towards the DNA, the β -barrel is closed by a lid consisting of two smaller sheets: one is formed by strands c', c, f, g, a, with strands c and c' reaching far down to the DNA. The second sheet is formed between strands e, e' and b (Fig. 2).

Although the main part of the T domain is formed by β -pleated sheets, helical segments are found in strand-connecting regions and at the carboxy terminus. The N-terminal strand A is immediately

followed by helix H1, helix H2 connects strands C' and E, and the last strand g leads into the C-terminal helices H3 and H4. Helices H3 and H4 are perpendicular to each other and they both interact with the DNA. Helix H3 is closely packed against helix H1, with hydrophobic side chains of both helices interdigitating. Both protein monomers show very similar conformations except for one loop (FG) which is involved in crystal contacts (r.m.s. difference $\alpha = 0.25$ Å without loop FG).

The dimer interface is formed predominantly by the ends of strand C', which pack against each other (Fig. 2b). Carbonyls from dimerically related Pro 125, Asp 126 and Pro 128 along the C' strands are bridged by water molecules, whereas atom N82 of Asn 129 forms a hydrogen bond to the carbonyl of Ser 127 of the other subunit. At the top of strands C', Pro 128 and Phe 130, assisted by Met 85 and Val 173, form a hydrophobic patch. Pro 128 adopts a *cis* conformation and is strictly conserved among different homologues. In contrast, Met 85, Phe 130 and Val 173 are jointly exchanged into Lys/Arg/His, Thr and Ala, respectively, at corresponding positions in some homologues (Fig. 1). This suggests that there may be two subgroups of T-domain proteins that can only homo- and heterodimerize within their subgroups. The relatively small dimer interface of 250 Å² is consistent with the observation that the protein is a monomer in solution (data not shown). The enhancement of DNA binding by the T protein in the presence of antibodies¹³ observed *in vitro* may be caused by stabilization of the dimer on DNA through the two antigen-binding sites of the antibody.

The T-domain structure reveals a novel sequence-specific DNA recognition architecture in which the protein contacts the DNA in both the major and minor grooves. Important contacts are made by residues protruding from loops and strands of the two small β -sheets

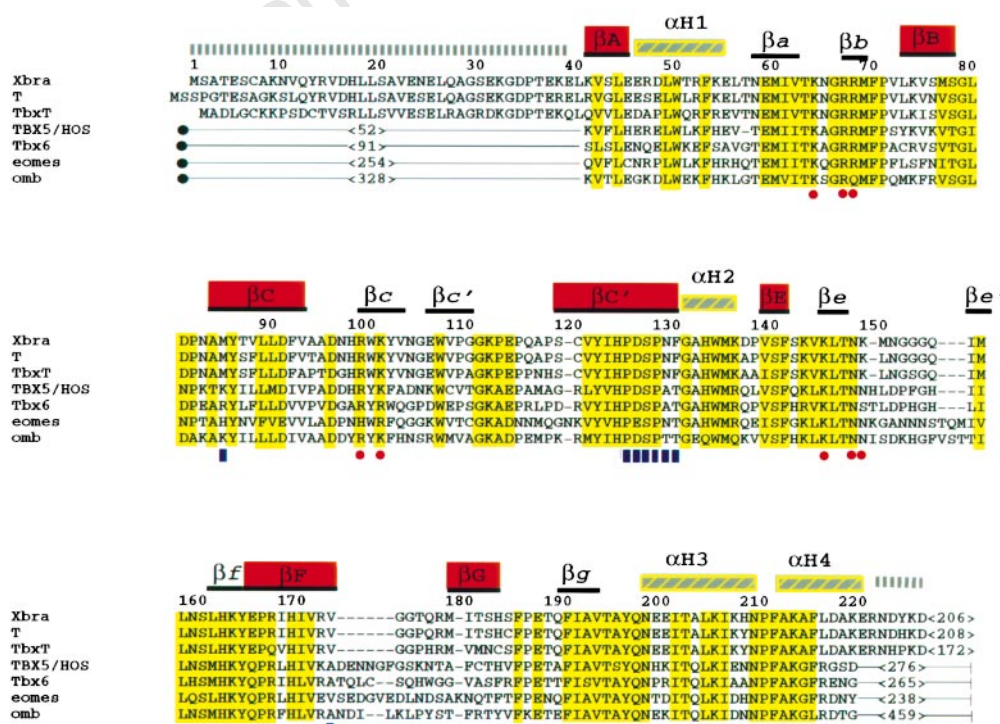


Figure 1 Sequence of the 226 amino-terminal residues of T.X. laevis (Xbra)² used for co-crystallization aligned with T mouse (T)¹, TbxT chick (TbxT)⁵, TBX5 human (TBX5/HOS)¹¹, Tbx6 mouse (Tbx6)⁴, eomesodermin (eomes)⁶ and optomotor-blind *Drosophila* (omb)¹⁰. The conserved N-terminal residues of T.X. laevis, T mouse and TbxT preceding the T domain are shown. For TBX5, Tbx6, eomesodermin and optomotor-blind, the number of residues before and after the T domain are given in brackets. Identical and conservatively substituted residues of the T domain are on a yellow background. Secondary structure

elements are indicated by lines (β -strands) and yellow hatched boxes (helices). Strands of the β -barrel related to Ig-domains are in red. The secondary structure assignment is based on visual inspection. Strands c, c' are connected by only one hydrogen bond to strand f, strands e, e' are connected by two hydrogen bonds to strand b. Black hatched bars indicate regions at the N and C termini that are disordered in the crystal. Blue rectangles and red circles indicate residues involved in dimerization and DNA binding, respectively.

bee' and *c'cfa*, and from residues of the two C-terminal helices H3 and H4 (Fig. 3a, b).

The two sheets *bee'* and *c'cfa* seem to act as a scaffold, which rigidifies the DNA-contacting residues and provides a continuous DNA-recognition surface: Residues in loop *ab* and strand *b* (Lys 64, Arg 67, Arg 68), strand *c* (Arg 99, Lys 101), strand *e* (Lys 145), loop *ee'* (Asn 148, Lys 149), loop *e'f* (Ser 160) and loop *gH3* (Thr 194, Tyr 196) form polar interactions with the DNA. They only contact the DNA backbone, except for Arg 67, which forms a direct hydrogen bond to N7 and a water-mediated hydrogen bond to O6 of guanine (bp5) in the major groove but also contacts the phosphate of the same base (Fig. 3a). There is an intimate hydrophobic contact between loop *gH3* and thymines of base pairs 8 and 9.

The C-terminal helices H3 and H4 recognize the DNA in the minor groove. Helix H3 bridges the DNA backbone and is positioned by phosphate contacts with Tyr 196 and Asn 209 at the beginning and end of the helix and by hydrophobic contacts between Ile 206 and the ribose of guanine (bp5). The N-terminal part of helix H4 points deeply into the minor groove forming hydrophobic contacts with backbone sugars and base edges. The aromatic rings of Phe 211 and Phe 215 in particular reach down to the bottom of the minor groove (Fig. 3a, b). Besides hydrophobic contacts, we also observed a hydrogen bond between the carbonyl of Phe 211 and one hydrogen from the N2 of guanine (bp3). The

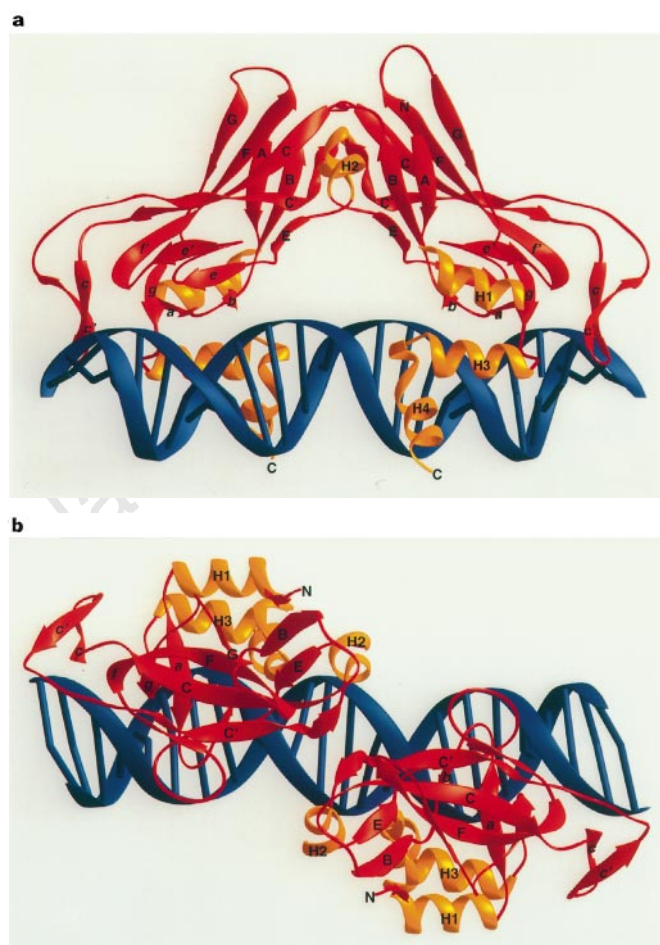


Figure 2 Ribbon diagram of the T-domain dimer bound to DNA. Depicted are residues 39–221 of both monomers (strands and loops: red, helices: yellow) and the 24-mer DNA duplex (blue). Labels mark strands of the central immunoglobulin fold (A–G) and the two smaller lid-forming sheets (*bee'* / *c'cfa*). This figure and Fig. 3b, c were generated with the program Ribbons³⁰. **a**, View perpendicular to the DNA axis with the dyad vertical; **b**, rotated by 90° around the DNA axis with respect to **a**.

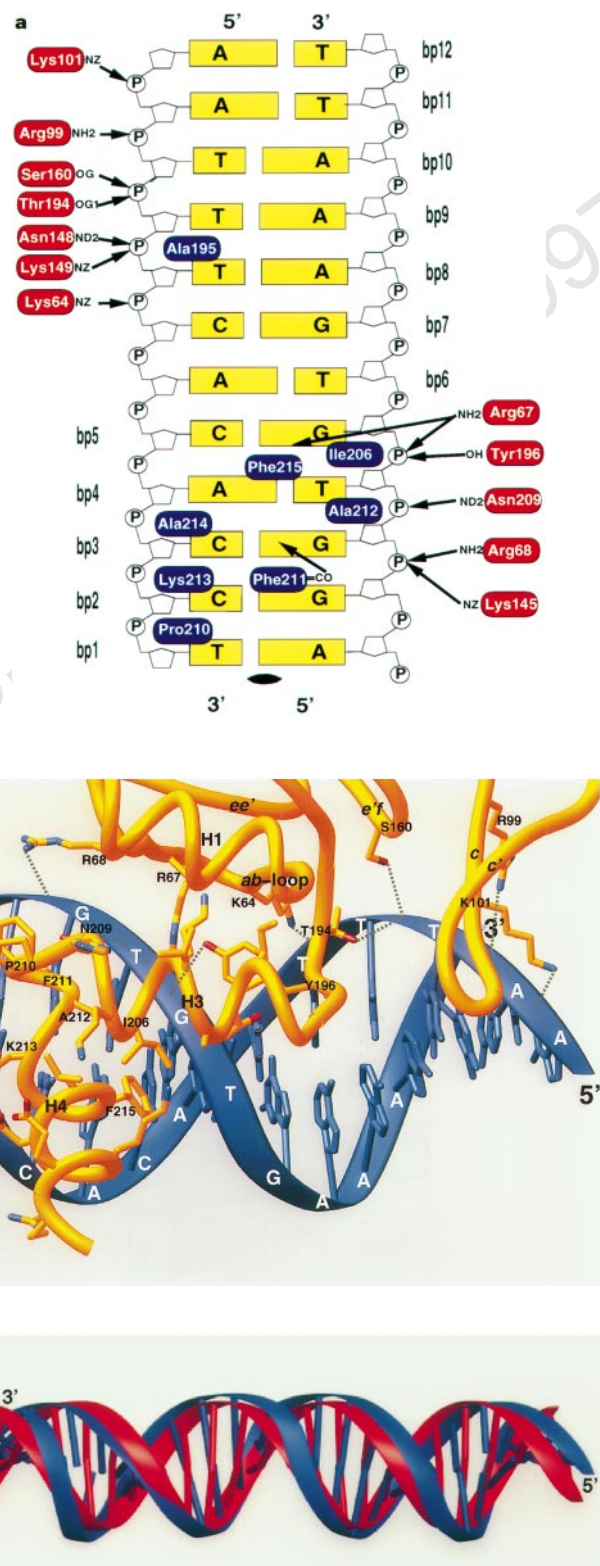


Figure 3 DNA recognition by the T domain. **a**, Protein–DNA interactions in one half-site. Polar interactions are indicated by arrows. Residues against a red background are involved in polar interactions; those against a blue background make hydrophobic contacts and are placed next to the region of DNA that they contact. The dyad generating the second half-site is indicated at the bottom. **b**, Interactions of one monomer with DNA. The view is the same as in Fig. 2a but with the contacts at the right half-site blown up. Polar interactions are indicated by dashed lines. For clarity, residues 145, 148 and 149, which also contact the DNA backbone, have been omitted. **c**, Superposition of the 24-mer DNA duplex found in the T domain–DNA complex (blue) and a 24-mer DNA duplex of regular B-form DNA (red). The orientation corresponds to the view in Fig. 2a.

carbonyl group of the protein backbone only accepts guanine at this position and is not affected by alterations in the amino-acid side chain which underlines its importance.

Helices H3 and H4 do not form a helix–turn–helix (HTH) motif: they are perpendicular to each other, connected by only two residues (HTH motif: helices crossing angle 120°, connected by a turn of four residues with glycine in position 2) and, most important, helix H4 interacts with the minor groove of the DNA, whereas in HTH motifs the helix–DNA interaction occurs in the major groove.

Comparing the T domain with structures deposited at the PDB data bank using programs Superimpose¹⁴ and Dali¹⁵, the similarity was greatest between the N-terminal domain of human NF-κB p50 (ref. 16; r.m.s._{73CA-atoms} = 1.94 Å) and the T domain, followed by proteins containing immunoglobulin folds and the tumour suppressor p53 (ref. 17; r.m.s._{73CA-atoms} = 2.05 Å).

The similarity of the N-terminal domain of NF-κB p50 and the T domain extends beyond the trivial similarity of proteins sharing an immunoglobulin fold. Superimposing the central β-barrels moves the AB loop of NF-κB p50, known as the 'DNA-recognition' loop, onto the *ab* loop of the T domain. In both domains, recognition in the major groove by arginines protruding from this loop and the relative orientation of the domains with respect to the DNA is maintained.

In the crystal, the 24-mer DNA duplex with blunt ends is not packed continuously but instead is packed at both ends against loops *cc'* of neighbouring molecules. The DNA in the complex is straight and adopts a B-form-DNA-like conformation, with an average rise of 3.3 Å and an average helical twist of 10.5 residues per turn. Despite its overall similarity to B-form DNA, there are marked differences (Fig. 3c): the minor grooves are markedly enlarged, with phosphate–phosphate distances of up to 10 Å across the minor groove (in B-form DNA, this width is 5.7 Å). The minor grooves are enlarged as a result of the extensive minor-groove contacts of the C-terminal helices and extend over 5 base pairs. Small adjustments of the DNA backbone allow the widening of the minor groove while keeping its depth comparable to that in B-form DNA. At the ends of the duplex, the A+T-rich sequences have a very narrow minor groove and large propeller twists.

As in other minor-groove-bound protein–DNA complexes, there is a substantial widening of the minor groove, with extensive hydrophobic contacts and few polar interactions. However, these interactions do not cause an overall bend in the DNA, which distinguishes this complex from other DNA complexes such as the TATA-box binding protein¹⁸, the repressors PurR¹⁹ and LacI²⁰, HMG proteins LEF-1²¹ and SRY²², and IHF²³. Two observations might explain these differences: first, we do not observe side chains intercalating between base pairs, in contrast to the complexes cited above; second, strong backbone contacts of each T monomer to one strand in the inner region and to the opposite strand in the outer region of each half-site might prevent DNA bending (Fig. 3a).

The T-domain structure and the observed protein–DNA interactions agree with most previously reported biochemical data: the T-domain dimer extends over the entire length of the 24-mer DNA duplex, as predicted from DNase I digestion experiments, and it contacts the phosphate backbone over a length of 22 base pairs, which is the minimum length of a DNA duplex required for a gel shift assay¹². Depurination and N7 methylation interference experiments identified guanine of base pair 5 as the main contact point in the major groove. This nucleotide forms a hydrogen bond with Arg 67 and it is the only base that directly makes contact in the major groove (Fig. 3b). The protection of thymine at base pairs 4 and 6 and the weaker one at base pairs 8 to 10 is due to steric hindrance of loops *ab*, *gH3* and *cc'*, which reach into the major groove. Our results disagree, however, with previous observations suggesting that the binding of the T domain to a palindromic recognition sequence is monomeric. We found that 50% of the DNA was free in gel-shift assays and gel filtration experiments using equimolar amounts of DNA-binding domain and DNA duplex, which is in agreement with the crystal structure analysis indicating that the T domain dimerizes upon DNA binding but is monomeric without DNA (data not shown).

The crystal structure explains the specificity for most of the nucleotides found in more than 85% of the sites isolated by *in vitro* selection¹² (underlined in the palindromic binding site AATTTCACACCTAGGTGTGAAATT used for co-crystallization): hydrogen bonding identifies G-C base pairs 3 and 5 of each half-site as important DNA specificity determinants (bold letters; see also

Table 1 Structure determination of the T domain–DNA complex

Table 1. Structure determination of the P domain- BM1 complex					
Data collection					
Data set	Resolution range (Å)	R_{sym} (%) [*]	X-ray source	Reflection Observed/unique	Completeness (%)
Native	20–2.5	3.6	BM 14 (ESRF)	101,653/22,983	98.9
Iodo-3†	25–2.9	13.9	Rotating anode	47,273/14,301	96.5
Iodo-8	25–2.9	10.2	Rotating anode	43,756/13,478	91.3
Iodo-9	25–2.9	8.4	Rotating anode	44,453/13,616	92.3
Se-methionine	20–2.5	5.2	BM 1 (ESRF)	82,541/21,495	92.9
Phasing statistics					
Data set	Isomorphous‡ difference (%)	Phasing power§ acentric/centric	$R_{\text{cullis}}^{\parallel}$ acentric/centric	Figure of merit acentric/centric/all	
Native	–	–	–	0.491/0.660/0.514	
Iodo-3	17.9	1.08/0.83	0.81/0.80		
Iodo-8	18.3	1.58/1.11	0.70/0.68		
Iodo-9	17.4	1.81/1.30	0.63/0.59		
Se-methionine	13.8	1.27/1.00	0.80/0.71		
Phasing statistics					
Resolution range (Å)	20–2.5				
Total number of non-hydrogen atoms	4,075				
Number of water molecules	143				
<i>R</i> -factor (%)	21.5 (for 20,637 reflections)				
R_{free} (%)¶	28.3 (for 2,262 reflections)				
R.m.s. deviation:					
Bond length (Å)	0.009				
Bond angles (deg)	1.41				

**R*_{sym} = $\sum_i |I(i, h) - \langle I(i, h) \rangle| / \sum_i I(i, h)$ where $\langle I(i, h) \rangle$ is the mean of the *i* observations of reflection *h*.

† Iodo-derivatives 3, 8 and 9 co-crystallized with oligonucleotide AATTT₂CACACCT₃AGGTGTGAAATT and contained iodo-uracil at positions T₁, T_{1,3} and T_{1,2}, respectively.

‡ Isomorphous difference = $\sum |F_{PH} - F_P| / \sum F_{PH}$; *F*_{PH} and *F*_P are the derivative and native structure factor amplitudes, respectively.

§ Phasing power is the mean value of the heavy-atom structure factor amplitudes divided by the lack of closure.

|| *R*_{cullis} is the mean lack of closure divided by the mean isomorphous difference.

¶ *R*_{free} is calculated from a subset of 10% of the data.

Fig. 3). T-A base pair 4 is specified by the side chain of Phe 215 (Fig. 3b). Its close contact with the adenine would not tolerate the exocyclic N2 or O2 of guanine or thymine/cytosine, respectively. Hydrophobic contacts between the thymine 5-methyl group in base pair 8 and loop gH3 provide further specificity.

The pronounced propeller twists of the central A-T base pairs 1/-1 and the marked roll observed between them may lead to further 'indirect' specificity. Certainly preferences for A+T-rich sequences are also created at the outer regions of the binding site, where we observe narrow minor grooves and strong propeller twists. Base pairs 6 and 7 were identified as less important specificity determinants in at least one of the half-sites and they are not directly contacted by the protein in the structure.

The greater sequence conservation of one half-site of *in vitro* selected target sites could indicate that one strong and one weak half-site are sufficient for DNA binding but a single half-site is not¹². Thus, *in vivo* target sites of T-domain proteins might not occur as palindromes, but rather as imperfect indirect repeats. In the context of a promoter, the T protein might associate with other factors. In such cases, the binding sites may also consist of T half-sites. Sequences identical in 8 to 9 positions to the half-site 5'-AGGTGTG AAA-3' occur in the upstream region of candidate target genes of mouse T protein (our unpublished results), but whether these are functionally relevant remains to be investigated. □

Methods

Crystallization and structure determination. The DNA sequence encoding residues 1-226 of T protein from *Xenopus laevis* was cloned in a T7 RNA polymerase expression system, pT7-7 (Novagen), and overexpressed in *Escherichia coli* BL21(DE3) cells at 37°C for 3 h. After cell lysis and polyethylenimine precipitation, followed by ammonium sulphate precipitation, the protein was purified by two steps of column chromatography (SP-Sepharose and Superose-12; Pharmacia). Selenomethionine-substituted protein was overexpressed in the methionine auxotroph strain DL21(DE3). To prevent oxidation, selenomethionine protein was purified in the presence of 10 mM DTT. Chemically synthesized oligonucleotides were purified by anion-exchange chromatography (Mono-Q; Pharmacia) at pH 12, dialysed against distilled water and lyophilized. Oligonucleotides containing iodo-uracil (Glen research) were protected from light during purification and crystallization.

For crystallization, equal volumes of 0.55 mM protein dimer in 0.1 M NaCl, 10 mM HEPES, pH 7.5, 1 mM DTT were mixed with previously annealed 0.6 mM DNA duplex in the same buffer. Crystals were grown by the hanging drop method over a reservoir containing 2-8% PEG8000, 20 mM MgCl₂, 50 mM sodium acetate buffer, pH 4.2, 1 mM DTT, 5 mM spermine. In general, 1 µl of complex was mixed with 1 µl of reservoir solution. Crystals of the T domain-DNA complex have a plate-like morphology and grew within 2-3 days to a size of 700 × 300 × 30 µm³. Crystals often grew as clusters, which then had to be dissected; the crystals were harvested in the reservoir buffer with 12% PEG. To collect data at cryogenic temperature, crystals were transferred stepwise into solutions containing an increasing concentration of glycerol to a final concentration of 25% (v/v) glycerol and frozen in a stream of nitrogen at 100K. All data were collected at 100K on a Mar Research image plate and processed with programs DENZO and SCALEPACK²⁴.

The selenomethionine data set was recorded at a wavelength of 0.87 Å (*f''*(Se) = 3.11). The crystal was aligned with one two-fold axis along the spindle to allow the simultaneous recording of Bijvoet pairs. The space group of the crystals is *P*₂₁₂₁, with cell dimensions of *a* = 37.9 Å, *b* = 113.9 Å, *c* = 149.0 Å. They contain one complex per asymmetric unit and diffract beyond 2.0 Å resolution using synchrotron radiation. The structure was solved by multiple isomorphous replacement combining data from three iodo-uracil DNA derivatives. Difference Patterson and difference Fourier synthesis allowed us to locate six independent iodine positions. A first multiple isomorphous replacement (MIR) map allowed us to determine the molecular envelope of each monomer with its DNA half-site. After twofold averaging, solvent flattening and histogram matching with program dm²⁵, an almost complete model was built into the improved electron density map. Model building was done by using program O²⁶. This model was confirmed by data

from a selenomethionine derivative, which showed strong peaks at the expected positions in isomorphous and anomalous difference Fourier maps phased with the three iodine derivatives. Of the nine methionines present in our construct, only the N-terminal methionine could not be located.

In the final heavy-atom refinement using program mlphare²⁵, all four derivatives and the anomalous signal in the Se-methionine data were included (Table 1). The resulting electron density map, again improved by program dm, was of excellent quality and enabled the model to be completed. Table 1 shows the statistics for data collection and phasing.

The current model has been refined with program X-PLOR²⁷ to a crystallographic *R*-factor of 21.5% (*R*_{free} = 28.3%; ref. 28) using data between 20-2.5 Å. It contains 143 water molecules. The geometry of the model is very good and no residues are seen in disallowed regions of the Ramachandran plot (92.4% in most favoured regions according to program PROCHECK²⁹).

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Correspondence and requests for materials should be addressed to C.W.M. (e-mail: mueller@embl-grenoble.fr). Atomic coordinates have been deposited with the Protein Data Bank (Brookhaven National Laboratory) under accession number 1XBR.

Neuroscience mardi gras

With some 25,000 participants descending on New Orleans, attendees of the Society for Neuroscience meeting must be wondering how to cover such an event. Here are some ideas on new products that may be on exposition.

Neuronavigation system

From Laser Lines

A system that will enable surgeons to improve the accuracy and safety of complex operations

Near-infrared cameras show surgeons the exact positions of surgical instruments both directly and where they might be employed on three-dimensional reconstructions of the patient's scans. Using CT head scans, the system shows on a television screen exactly where the instruments are inside the head with a virtual reality image that depicts where the surgeon is operating. The system incorporates ceramic balls on the instruments for detection by the camera. CAT and MRI scans, as well as images from microscopes, endoscopes or intracranial ultrasound can also be incorporated.

Reader Enquiry No. 100

Ischaemia models for drug discovery

From Cerebrus

A range of new in vivo stroke models to help identify potential new therapeutic agents and show their efficacy

Cerebrus is working on novel treatments for ischaemia, and has both well established models of ischaemic stroke (permanent or transient middle cerebral artery occlusion and four vessel occlusion), and models that are exclusive to the company, such as mouse cerebral embolism, haemorrhagic stroke and photochemically-induced ischaemia. The models should provide pharmaceutical companies with relevant information about the efficacy of candidate drugs to advance drug discovery programmes, while freeing up valuable 'in-house' resources.

Reader Enquiry No. 101

Brain/tissue slice chamber

From Medical Systems

The chamber features a single base unit and two interchangeable top units

The Haas-top configuration utilizes semi-



The brain slice chamber with Haas and Zbicz-based design features.

submersion techniques, providing excellent stability for intracellular recording and rapid exchange of perfusion fluids. The Zbicz top is designed to maintain viable slices of tissue submerged under a constant flow of perfusing liquid. Therefore, alternative techniques can be employed for evaluation of drug actions. Cell life of ten or more hours is possible. A companion temperature controller (model TC-202A) is offered, which functions with either top chamber and regulates temperature between 30–45 °C ± 0.2 °C.

Reader Enquiry No. 102

CM 1850 cryostat

From Leica

Designed for simple operation and a high-volume workload in a variety of routine histology and clinical pathology sectioning

The CM 1850 features easy disinfection of the splash-proof microtome and an enclosed drainage system, which reduces the release of bacteria and germs into the air while sectioning fresh and potentially infectious tissue specimens. The vacuum-panel insulation design leads to stated power savings of about 10 per cent. This enhances the durability of the refrigerating system and ensures stable cryochamber temperatures. The optional Peltier cooling stage provides rapid specimen freezing down to –60 °C and, used in combination with the 10-disk capacity, actively cooled freezing shelf (to –45 °C) and heat extractor system, provides a very rapid freezing system for specimen preparation. The cryostat is equipped with temperature sensors for testing refrigeration temperatures. The sensors are easily accessible, as are all of the serviceable components. Status of both refrigeration and electronic systems can be checked via an external PC link.

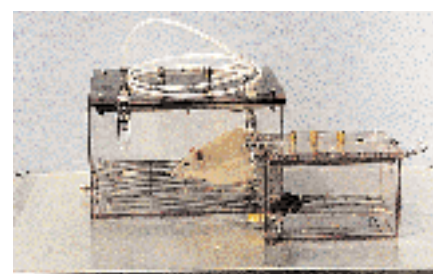
Reader Enquiry No. 103

Air-tight animal cages

From Columbus Instruments

Animal cages made of high-density, crash-resistant 0.634-cm transparent plastic Lexan

Each cage is equipped with stainless steel latches and a suspended stainless steel floor grid. Rigid construction and an airtight seal around the top lid make them ideal for testing animal oxygen consumption and exposure to other gaseous environments. Connectors for plastic 0.634-cm hoses facilitate gas/air delivery and sampling. Two standard models are available: 31 cm × 19 cm × 18 cm for rats and 20 cm × 10 cm × 12 cm for mice. On request, special cage sizes can



No quantum cats in these air-tight boxes — clear Lexan allows easy viewing of mice and rats.

be made to the customers' specifications. The new animal cages can be used for testing animal behaviour and physiology when exposed to environmental pollutants, as well as for measuring animal oxygen consumption and respiration rate measurements.

Reader Enquiry No. 104

EPC-9 digital patch-clamp amplifier

From ALA Scientific Instruments

The amplifier comes complete with a built-in digital oscilloscope, a pulse generator, an analog filter and a data acquisition system

Features include full computer control (PC- and Mac-based), automatic selftest and calibration, automatic capacitance neutralization up to 1,000 pF, capacitance tracking, variable high-quality filters, automatic leak subtraction, and currents up to 2 µA. The EPC-9 permits rapid switching from current clamp to voltage clamp mode without transients. Amplifier noise is as quiet as capacitative headstage-type amplifiers. Types of experiments that can be conducted include single-channel recordings, whole-cell voltage clamp studies, current clamp studies, artificial membrane recordings, and loose patch type recordings. The EPC-9n double/triple patch clamp unit is now available in one chassis. When combined with Pulse software, EPC-9 the becomes a more flexible and advanced patch-clamp system. Pulse now supports Digidata 1200A.

Reader Enquiry No. 105

Reagents

Neuronal glutamate transporter (EAAC1) polyclonal antibody

From Chemicon

Chemicon's new goat polyclonal antiserum to the neuronal glutamate transporter offers unique possibilities to localize glutamatergic neurons in the nervous system

The antibody has been used successfully for immunohistochemistry and works on rat, mouse and guinea pig. It is expected to work



Chemicon's new goat polyclonal antiserum to the neuronal glutamate transporter.

on human, rabbit and monkey due to peptide sequence homology. As the antiserum is generated in goat, it may be used in double-labelling experiments.

Reader Enquiry No. 106

$\alpha 4\beta 2$ nAChR ligand

From RBI

A receptor ligand that is manufactured and sold with the exclusive permission of Abbott Laboratories

The classic nicotinic acetylcholine receptor (nAChR) is an ion channel protein consisting of $\beta 1$, δ , γ (ϵ in adult) and two $\alpha 1$ subunits. Various nAChR gene products exist in the peripheral and central nervous systems and these assemble to form receptors that have been implicated in a variety of responses, including analgesia and cognition. The analgesic ($\pm$)epibatidine has been identified as a potent ligand for the $\alpha 4\beta 2$ nAChR subtype, but it also displays significant activity at neuromuscular and ganglionic-like nAChRs. As well as being a potent ligand at the $\alpha 4\beta 2$ and ganglionic-like nAChR subtypes, $\alpha 4\beta 2$ nAChR can differentiate between the two major α -bungarotoxin-sensitive nAChRs ($\alpha 7$ and $\alpha 1$, $\beta 1$, δ and γ) by displaying marked selectivity towards the $\alpha 7$ subtype. Also offered is a selective D1 dopamine receptor agonist. This isochroman derivative is an agonist both *in vitro* and *in vivo*. When tested in marmosets treated with MPTP to induce a parkinsonian-like state, it induces locomotor activity and reduces the severity of the parkinsonian-like symptoms. The compound is active following either oral or subcutaneous administration.

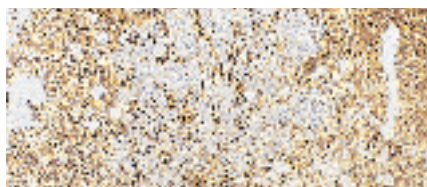
Reader Enquiry No. 107

Anti-NGFR MAb

From Dako

Monoclonal anti-human nerve growth factor receptor (NGFR), Clone NGFR5

This antibody recognizes the 75K NGFR, p75^{NGFR}. This p75 receptor protein is a member of the nerve growth factor/tumour necrosis factor superfamily of receptors, many of which function as mediators of cell death, or apoptosis. *In vitro*, unbound p75^{NGFR} has been demonstrated to promote neural cell death, whereas binding of p75^{NGFR} by NGF ligand or antibody has been shown to inhibit p75^{NGFR}-induced cell death. Immunohistochemical



Dako now offers an antibody that recognizes the 75K NGFR, p75^{NGFR}.

analysis with this antibody can be performed on routinely processed tissue sections, frozen sections or cell smears.

Reader Enquiry No. 108

Voltage-gated Ca²⁺-channel antibodies

From TCS Biologicals

A collection of voltage-gated Ca²⁺-channel antibodies under the Alomone Labs label

The new antibodies include polyclonal antibodies to the α_{1A} , α_{1B} , α_{1C} and α_1 subunits. The α_1 subunit forms a channel that resembles Q- and P-type voltage-gated Ca²⁺ channels. The new polyclonal antibody to the rat α_{1A} subunit recognizes both the major (190 K) and minor (210 K) forms of the subunit. The α_{1B} subunit forms the N-type voltage-gated Ca²⁺ channel, which is insensitive to dihydropyridines and blocked by ω -conotoxin GVIA and ω -conotoxin MVIIA. This polyclonal antibody recognizes both the 210 K and 240 K forms of the rat α_{1B} subunit. The α_{1C} subunit forms the cardiac and one of the brain L-type voltage-gated Ca²⁺ channels. Anti- α_{1A} recognizes both the 190 K and 210 K forms of rat α_{1C} . Anti-pan α_1 is raised against peptide CP corresponding to highly conserved residues of α_{1S} of skeletal muscle voltage-gated calcium channels containing N-terminal cysteine. It recognizes all types of α_1 subunits, cross-reacts with mouse and rat and is fully inhibited with soluble CP.

Reader Enquiry No. 109

QCB Select

From Quality Control Biochemicals

Antibodies for the selective detection of A β [1-40], [1-42] and [1-43]

QCB has developed three C-terminal specific, affinity purified rabbit polyclonal A β antibodies, which allow for the quantitative determination of A β [1-40], [1-42] and [1-43]. Selectivity for these antibodies is 300–500-fold and sensitivity in sandwich ELISAs is 5–15 pg for A β [1-40] and [1-43] and 15–25 pg for A β [1-42]. Other applications include RIAs, dot blots and immunohistochemistry procedures. An antibody directed against amino acids 17–28 of A β is also available.

Reader Enquiry No. 110

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal. □

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